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Signalling Pathways Downstream of Cadherin

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

December 2006

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ABSTRACT

Formation of cadherin-based adhesive structures at sites of cell-cell contact leads to activation of several signalling pathways inside the cell. In order to examine the molecular mechanisms involved in these pathways, my project focused on two binding partners of cadherin, p120 catenin and Hakai.

p120 catenin (p120) is an Armadillo family member, which binds to the cytoplasmic tail of cadherins at sites of cell-cell contact. It has been implicated in regulation of cell-cell adhesion by modulating cadherin trafficking, and also in the regulation of actin dynamics through its association with members of the Rho-GTPase family. The presence of p120 in the nucleus observed for a number of cell lines indicates an additional nuclear function for this protein. A number of biochemical assays show that p120 can bind to the transcription factor Glis2. In addition, p120 expression can induce C-terminal cleavage of the Glis2 protein. This cleavage is increased by the overexpression of Src, suggesting a role for Src-mediated tyrosine phosphorylation of p120. Since Glis2 has been shown to have a role in neuronal differentiation, an investigation was carried out on the effect of overexpressing Glis2 in the neural tube of developing chick embryos. Interestingly, overexpressing Glis2 inhibited neuronal differentiation. A similar effect was also observed when the cleaved form of Glis2 was co-expressed with p120. These results suggest a nuclear role for p120 by modulating the cleavage of the transcription factor Glis2, and potentially altering the transcription of genes in the neuronal differentiation pathway.

Hakai is an E3 ubiquitin ligase for the E-cadherin complex, which competes for binding on the E-cadherin intracellular tail with p120. As Hakai is ubiquitously expressed, it is likely that Hakai has other substrates in the cell. Using the results of a previously completed yeast two-hybrid screen, several potential binding partners for Hakai have been identified.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Multicellular organisms maintain their shape through regulated adhesion between cells and tissues. Embryogenesis and morphological changes throughout the life of the organism rely on regulated and selective contact between cells. For example, dorsal closure during gastrulation requires the bringing together of epithelial edges and formation of new permanent adhesions to form the dorsal midline (described for *Drosophila* by (Martin and Parkhurst, 2004). Tissue repair after wounding also requires coordinated movement of sheets of cells, and the subsequent re-establishment of cell-cell adhesion. Homeostasis in adult tissues requires the turnover of rapidly growing tissues, such as the lining of the gut, without compromising the barrier function of the epithelium. In addition, the maintenance of stable tissue organisation prevents the dissociation and spread of tumour cells.

Sites of cell-cell contact are called junctions, which function to stably and specifically connect neighbouring cells. Junctions are also important nexus points for cell-cell signalling pathways. Contacts between cells are not static, but rather are actively regulated to allow the cell to sense the environment it is in and respond to cues to proliferate, migrate, differentiate, apoptose, or maintain the current physiological state.

Electron micrographs from the 1960s showed the existence of three types of junction between epithelial cells (Figure 1.1;(Farquhar and Palade, 1963). Tight junctions (the *zonula occludens*) are the most apical of the junctional complexes, forming a 'seal' between the apical and baso-lateral membrane domains, resulting in obliteration of the intercellular space (Farquhar and Palade, 1963). This boundary formed by the tight junction helps maintains cell-surface polarity and also forms a semipermeable diffusion barrier between neighbouring cells (reviewed in (Anderson, 2001).

Adherens junctions (the *zonula adherens*) are positioned directly below the tight junction and are characterized by 'strict parallelism' of adjoining cell membranes, with an intercellular space of approximately 200 Å (Farquhar and Palade, 1963). The transmembrane core protein of the adherens junction is named cadherin (lower

schematic, Figure 1.1), and the intercellular space is filled by the extracellular domains of cadherins, which form homophilic *cis* and *trans* dimers (Gooding et al., 2004).

Desmosomes (the *macula adherens*) are characterized by an intercellular distance of approximately 240 Å and are not arranged as regularly as tight junctions (TJs) and adherens junctions (AJs). The desmosomes were predicted to have a 'button-like' type of attachment as opposed to the 'belt-like' attachment of TJs and AJs, which form a continuous ring of attachment around the cell (Farquhar and Palade, 1963).

Elements of the cytoskeleton are associated with each type of junction, conferring mechanical strength to cell-cell contacts and integrating adhesion with such diverse processes as polarity, motility, differentiation and morphogenesis (reviewed in (Balda and Matter, 2003; Gumbiner, 1996; Knust and Bossinger, 2002). In addition to this 'epithelial junctional complex', gap junctions form intercellular pores that directly connect adjacent cell cytoplasms, allowing the passage of hydrophilic molecules up to 1 kilodalton (kDa) (Evans et al., 2006).

Although all three members of the epithelial junctional complex are functionally significant for the signalling processes mentioned above, a particular focus has been put on the adherens junction, as recent evidence shows they are crucial sensors of extracellular stimuli and regulators of cellular dynamics. The molecular and regulatory mechanisms governing AJ signalling are not yet fully understood, and examination of the components of AJs is key to delineating these pathways. This chapter sets out what is known about signalling downstream of AJs, particularly with regard to two binding partners of cadherin. p120 catenin and Hakai both bind to the juxtamembrane domain of cadherin and their signalling roles are investigated in this thesis.

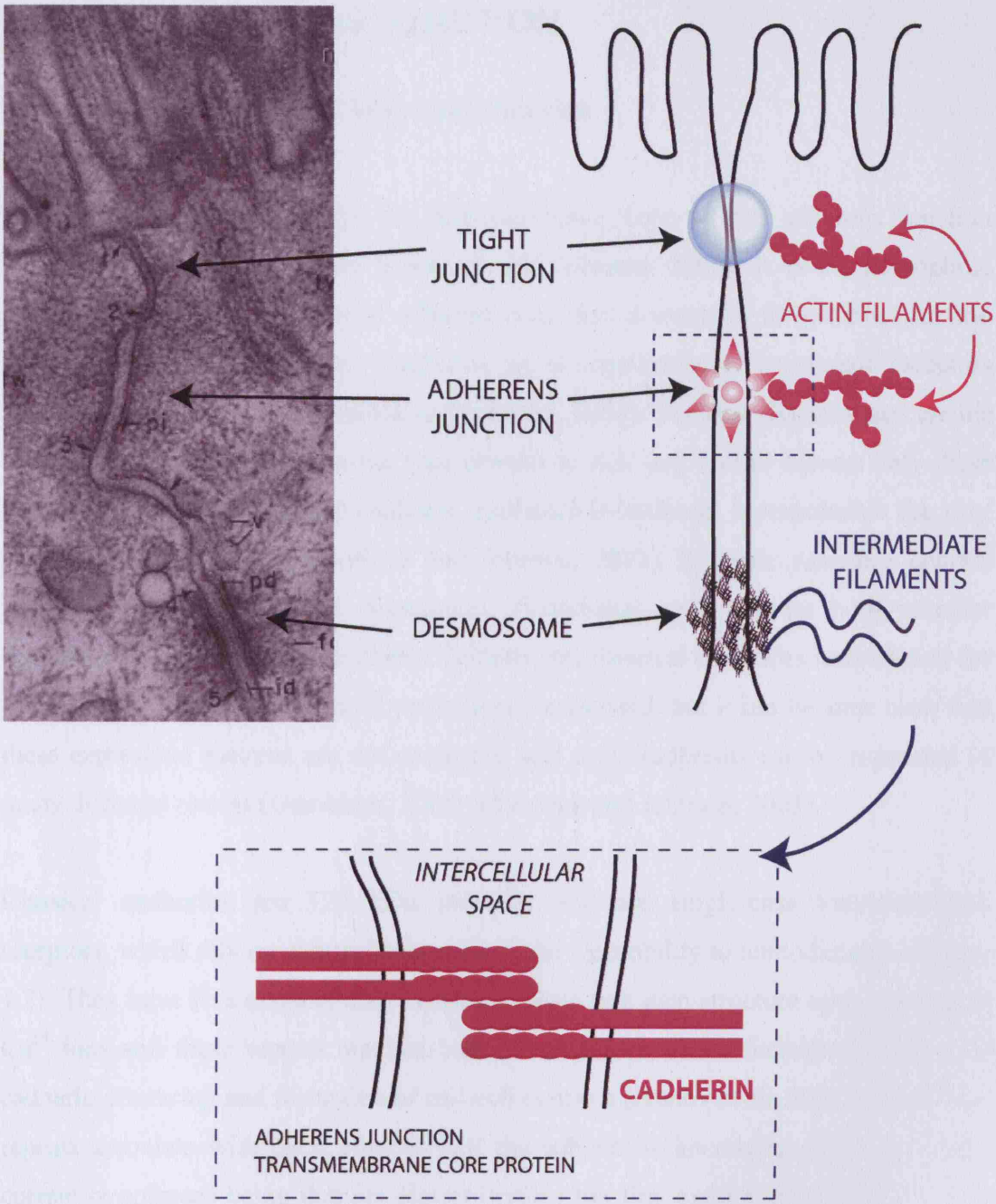


Figure 1.1 The epithelial junctional complex. *The electron micrograph is from the 1963 paper by Farquhar and Palade describing the characterization of the Zonula Occludens (Tight Junction), Zonula Adherens (Adherens Junction) and Macula Adherens (Desmosome) in various epithelia. The schematic shows that the electron dense 'fuzz' on the intracellular sides of the junctions is due to the association of elements of the cytoskeleton with each of the junctional complexes. Blowup of the Adherens Junction schematic shows that cadherin is the transmembrane core protein of the Adherens Junction.*

1.2 THE ADHERENS JUNCTION

1.2.1 Components of the Adherens Junction

The glycoprotein cadherin is the transmembrane core of the adherens junction (reviewed in (Takeichi, 1990; Wheelock and Johnson, 2003). It is the homophilic interaction between cadherins of adjacent cells that is essential for establishing and maintaining cell-cell adhesion. Cadherins are a superfamily of membrane receptors with over 100 members (Wheelock and Johnson, 2003). The best characterized are the classical cadherins, which are the type present in AJs, and I shall discuss only these here. The prototypic classical cadherin, epithelial E-cadherin, is responsible for very tight cell-cell adhesion (Wheelock and Johnson, 2003). Synaptic junctions contain both E-cadherin and neural N-cadherin. Endothelial cells express both vascular specific VE-cadherin and N-cadherin. Initially, the classical cadherins were named for the tissue in which they are most prominently expressed, but it has become clear that these expression patterns are not exclusive, and most cadherins can be expressed in many different tissues (Gumbiner, 2005; Wheelock and Johnson, 2003).

Classical cadherins are 120 kDa proteins, and are single-pass transmembrane receptors, which rely on extracellular calcium for their ability to homodimerise (Figure 1.2). They have five extracellular cadherin repeats that gain structure upon binding to Ca^{2+} ions and these repeats mediate both *cis* and *trans* dimer formation, leading to cadherin clustering and formation of cell-cell contacts (Ivanov et al., 2001). How these repeats associate with each other is still the subject of interesting structural work; current hypotheses being that *cis* dimers involve the first extracellular cadherin (EC) repeat, and *trans* dimer formation involves three or more EC domains from each of the two opposing molecules (Gooding et al., 2004). The transmembrane portion of cadherin is characteristic of that for Type I single-pass transmembrane proteins (Wheelock and Johnson, 2003). The intracellular tail of cadherin is extremely well conserved, and is the site of binding of a family of proteins called catenins, which link the AJ to the actin cytoskeleton (Ivanov et al., 2001). Figure 1.2 shows the cadherin-catenin complex.

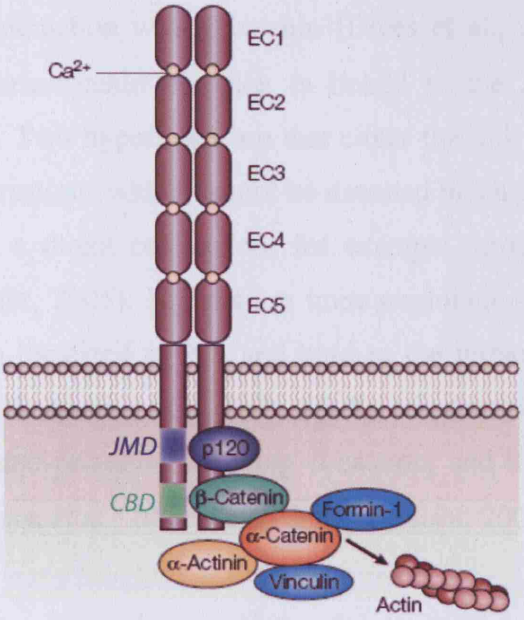


Figure 1.2 The cadherin-catenin complex. *Cadherin is a* cis *homodimer, and the five extracellular (EC) domains are bound together by Calcium ions (yellow circles) that confer domain rigidity for homophilic* trans*-dimer formation. The intracellular tail of cadherin has two highly conserved domains, the Juxtamembrane Domain (JMD) and the Catenin Binding Domain (CBD). p120 catenin binds to the JMD and β-catenin and plakoglobin bind to the CBD. β-catenin binds to α-catenin, which can also bind to actin and actin-binding proteins. This leads to the association of the cadherin-catenin complex with the actin cytoskeleton. Schematic adapted from Gumbiner, Nature Reviews Molecular Cell Biology, 2005.*

The Armadillo family proteins β-catenin or its close relative plakoglobin[1] associate directly with a core region of 30 amino acids within the C-terminus of the cadherin cytoplasmic domain (Jou et al., 1995; Stappert and Kemler, 1994; Zhurinsky et al., 2000). Binding of β-catenin stabilises the structure of the cadherin intracellular tail (Huber et al., 2001) and provides the platform for linking the cadherin complex to the actin cytoskeleton, conferring mechanical strength on the AJ (Jamora and Fuchs, 2002). β-catenin can bind simultaneously to cadherin and α-catenin, a protein involved in actin dynamics. α-catenin can interact with a number of actin re-modelling factors

[1] Plakoglobin (also known as γ-catenin) shares common protein partners with β-catenin and can fulfil some of the same functions, but cannot compensate for defects in Wnt signalling resulting from a lack of β-catenin (Zhurinsky et al., 2000). Thus, subsequent remarks in this Introduction about β-catenin function at the AJ can be taken to include plakoglobin, but the description of the Wnt signalling pathway (Section 1.2.2) refers only to β-catenin.

(Kobielak and Fuchs, 2004), and can also bind directly to actin, but in a mutually exclusive way to its interaction with β-catenin (Drees et al., 2005; Yamada et al., 2005). How the cadherin-catenin complex is linked to the actin cytoskeleton is currently under review. Two hypotheses are that either the link is mediated by many weak and transient interactions which cannot be detected biochemically; or, that other AJ molecules mediate a direct connection, for example through the nectin-afadin system (Gates and Peifer, 2005). Nectins are immunoglobulin-superfamily adhesion molecules that are also localized to AJs and bind to the intracellular protein afadin (Mandai et al., 1997; Takahashi et al., 1999). Afadin can also bind to actin as well as to several actin-associated proteins, including α-catenin, and thus could serve as an alternative link (Tachibana et al., 2000; Takai and Nakanishi, 2003).

p120 catenin (from hereon referred to as p120), a β-catenin related protein, binds to the juxtamembrane domain (JMD) of the cadherin intracellular tail (Reynolds et al., 1994; Shibamoto et al., 1995), in contrast to β-catenin which binds to the so-called catenin-binding domain (CBD) (Figure 1.2; (Stappert and Kemler, 1994). Both p120 and β-catenin bind to cadherin through their Arm repeat domains, a structural motif important for protein-protein interactions (Daniel and Reynolds, 1995; Hulsken et al., 1994). p120 does not bind to α-catenin and cannot function in place of β-catenin (Daniel and Reynolds, 1995). The role of p120 in the cadherin complex is described further in Section 1.3.

1.2.2 Signalling roles of the cadherins

Cadherins have been implicated in a number of signalling pathways. Evidence for this role in signal transduction came initially from developmental studies and investigations of epithelial carcinomas. Subsequently, several functional interactions between cadherins and the Rho family of small GTPases have been reported. In addition, it also seems that cadherins can directly affect each other's function, with forced expression of N-cadherin leading to downregulation of E- and P-cadherin in epithelial-like squamous cells (Islam et al., 1996). The roles of cadherin in Wnt,

receptor tyrosine kinase (RTK), and Rho-family signalling pathways are described briefly below.

The Wnt pathway

After the identification of β-catenin as a component of AJs, sequencing revealed that it is a close homologue of the Drosophila segment polarity gene *Armadillo* (McCrea et al., 1991), suggesting a role for β-catenin in development. Indeed, the role of β-catenin in the Wnt signalling pathway during development is now well characterized (Brembeck et al., 2006). Wnt pathways are involved in the control of gene expression, cell behaviour, cell adhesion, and cell polarity (reviewed in (Cadigan and Liu, 2006; Hlsken and Behrens, 2000; Moon et al., 2002). Wnt is an extracellular matrix-associated growth factor that interacts with its receptors, Frizzled/LRP (low-density-lipoprotein-related protein), to initiate a signal transduction pathway that stimulates synthesis of proteins involved in cell growth, such as cyclin D1 and myc (Hlsken and Behrens, 2000). Intracellularly, Wnt signalling leads to stabilisation of cytosolic β-catenin.

As shown in Figure 1.3, in the absence of Wnt, cytosolic β-catenin is complexed with a scaffold of Axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β), leading to phosphorylation of β-catenin, and its subsequent targeting for ubiquitination and degradation in the proteasome. When Wnt is present, its co-receptors Frizzled/LRP are able to signal to a scaffold protein Dishevelled, which becomes phosphorylated and then interacts with the Axin/APC/GSK3β complex. This inhibits GSK3β-phosphorylation of β-catenin. Stabilised β-catenin can then enter the nucleus, and associates with T-cell factor (TCF) transcription factors, which leads to the transcription of Wnt-target genes. When β-catenin is absent, certain TCFs repress transcription by interacting with the co-repressors Groucho and CtBP. (Summarised from the online database WntWorld: www.gcd.med.umn.edu/millerlab/Wnt/wntworld and this topic is reviewed in (Hlsken and Behrens, 2000).

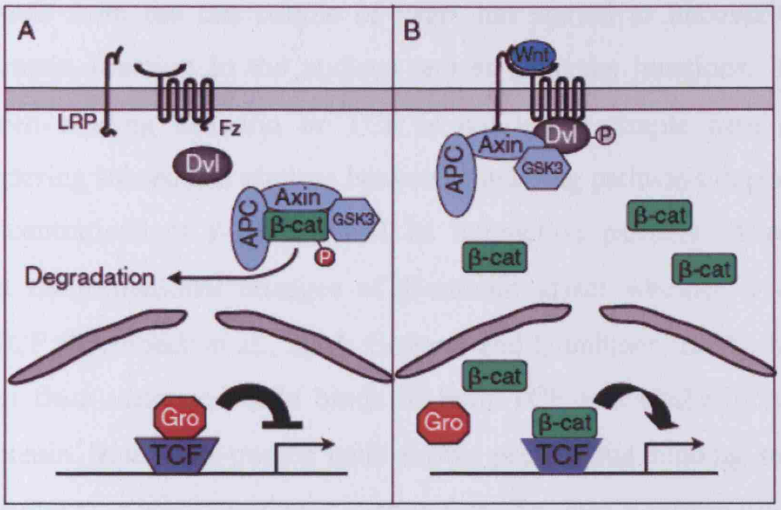


Figure 1.3 The Wnt signalling pathway. A. *In the absence of Wnt, β-catenin is either degraded via the action of the APC/Axin/GSK3β complex after phosphorylation by GSK3β, or sequestered at the cadherin-catenin complex (not shown). Wnt target genes are transcriptionally repressed by binding of Groucho (Gro) and other corepressors to TCF.* ***B.*** *Wnt binding to Fz-LRP receptors leads to inactivation of the APC/Axin/GSK3β complex, through a combination of LRP-Axin association and Dishevelled (Dvl) phosphorylation blocking the action of GSK3β. Stabilized β-catenin can enter the nucleus to cause derepression of Wnt target genes by binding to TCF, converting it to a transcriptional activator. Schematic from Cadigan and Liu, J Cell Sci, 2006.*

A key observation that identified a role of the cadherin-catenin complex in epithelial growth control came with the identification of the APC gene (Powell et al., 1992), which is altered in individuals with adenomatous familial polyposis, as well as in some sporadic colon cancers, as it was then shown that β-catenin binds to the APC gene product (Rubinfeld et al., 1993; Su et al., 1993). Impairment of the APC protein activity that targets β-catenin for degradation can lead to increased signalling through the Wnt pathway due to the presence of elevated levels of cytosolic β-catenin (Moon et al., 2002; Munemitsu et al., 1995). In addition, it has been observed that in a number of tumour cell lines with little or no cadherin expression, β-catenin signalling through the Wnt pathway is elevated leading to higher proliferative capacity (Moon et al., 2002; Munemitsu et al., 1995). This indicates that cadherin can function to limit Wnt signalling by sequestering β-catenin at cell-cell junctions.

Interesting work from the last couple of years has started to uncover the balance between β-catenin function in the nucleus and at adherens junctions. It seems the choice between binding cadherin or TCF is not just a simple case of differing affinities, rendering subsequent choices between signalling pathways dependent purely on local concentrations of β-catenin and its interaction partners. A recent report indicates that conformational changes of β-catenin affect whether it can bind to cadherin or TCF (Brembeck et al., 2004; Gottardi and Gumbiner, 2004). It was shown that β-catenin from untreated cells binds to both TCF and cadherin equally well, whereas β-catenin from Wnt-treated cells shows preferential binding to TCF. This appears to be due to a conformational change from an open conformation, where all Arm repeats are available for binding (necessary for cadherin binding) to a closed conformation, where the C-terminus folds back on the Arm repeats, favouring the interaction with TCF (Gottardi and Gumbiner, 2004).

Signalling through Receptor Tyrosine Kinases

Numerous reports indicate that classical cadherins interact with RTKs (Wheelock and Johnson, 2003). N-cadherin has been shown to facilitate the dimerization of the fibroblast growth factor (FGF) receptor to initiate a signalling pathway during neurite extension (Doherty et al., 2000; Skaper et al., 2001). Other studies suggest that N-cadherin may influence tumour cell behaviour through interactions with the FGF receptor. Invasive epithelial-derived tumours with aberrant N-cadherin expression can be suppressed by the addition of inhibitors of FGF receptor signalling (Kim et al., 2000). The fourth extracellular domain of N-cadherin is necessary and sufficient for this activity (Kim et al., 2000). Similarly, links between E-cadherin and the epithelial growth factor (EGF) receptor, and VE-cadherin and the vascular endothelial growth factor (VEGF) receptor have been reported. E-cadherin can induce ligand-independent activation of the EGF receptor and subsequent activation of MAP kinase (Pece and Gutkind, 2000). In contrast, a ligand dependent association between the VEGF receptor, VE-cadherin, β-catenin and phosphatidylinositol 3-kinase (PI3K) exerts a negative effect on growth factor signalling (Carmeliet et al., 1999; Zanetti et al., 2002). In addition, a recent paper shows how this interaction between VEGF signalling and VE-cadherin also impacts on the integrity of the AJ. A pathway from VEGF ligation to VEGF Receptor-2 leads to phosphorylation of the VE-cadherin intracellular tail, and

recruitment of β-arrestin, ultimately causing endocytosis of VE-cadherin and an increase in vascular permeability (Gavard and Gutkind, 2006). Clearly, the propagated signals from cadherins' associations with RTKs are both cadherin-dependent and cell-context dependent.

Signalling through Rho GTPases

Interest in how Rho GTPases might function in cell-cell adhesion came first from the observation that Rho, Rac and Cdc42 are localized to cadherin based cell-cell contacts. Further, the formation of cadherin-mediated cell-cell contacts can activate Rho, Rac and Cdc42 (reviewed in (Braga, 2000; Braga, 2002; Yap and Kovacs, 2003). These proteins are members of the Rho family of small GTPases. GTPases are transducers of many signalling pathways in the cell, and act by cycling between an inactive GDP-bound form, and an active GTP-bound form, in which the GTPase can bind to downstream effectors (reviewed in (Etienne-Manneville and Hall, 2002). Multiple regulators that facilitate GTP capture (guanine exchange factors, GEFs), or accelerate GTP hydrolysis (GTPase-activating proteins, GAPs), or sequester GTPases in the GDP-bound form (GDP dissociation inhibitors, GDIs) are responsible for the tight temporal and spatial control of GTPase activity (Etienne-Manneville and Hall, 2002). The intimate relationship between this family of GTPases and the actin cytoskeleton is very well documented, and as such, Rho GTPases are clearly implicated in the formation, maintenance and disassembly of AJs due to the attachment of microfilaments to these junctions. This is a vast and rapidly moving field of research; since the Rho GTPases are not the focus of this thesis, two illustrative examples are given here to highlight the role of Rho GTPases in AJ signalling.

Firstly, both the establishment and maintenance of cadherin-dependent cell-cell contacts are reliant on Rho and Rac activity, as demonstrated by experiments in keratinocytes (Braga et al., 1997). Blocking endogenous Rho or Rac, using C3 toxin or dominant negative Rac, respectively, removed cadherin complexes from junctions. Moreover, the recruitment of actin to sites of nascent cell-cell contact is dependent on the presence of functional E-cadherin receptors and endogenous Rac (Braga et al., 1997). Thus, signalling to the actin cytoskeleton upon ligation of cadherin is transduced through Rho and Rac, and this cytoskeletal reorganisation is necessary for maintenance of cadherin-based junctions.

As a second example of Rho GTPase signals functioning to stabilise the cadherin-catenin complex, the Kaibuchi group demonstrated a role for Rac and Cdc42 in regulating the function of IQGAP (a RasGAP related protein) (Fukata et al., 1999; Kuroda et al., 1998; Weissbach et al., 1994). IQGAP binding to β-catenin leads to destabilisation of the cadherin-catenin complex and downregulation of adhesion (Fukata et al., 1999). However, in the presence of active Rac or Cdc42, IQGAP was inhibited from binding β-catenin, leading to stabilisation of the cadherin-catenin complex (Fukata et al., 1999). What signal is upstream of Rac or Cdc42 in this system is not clear, although other work indicates that PI3K and EGF signalling are required for Rac activity following cadherin engagement (Wheelock and Johnson, 2003). In addition, it is possible to hypothesize that when nascent cell-cell contacts are being formed, the concomitant recruitment and activation of Rho GTPases to junctions, as described above, is sufficient to inhibit IQGAP while cadherin-based contacts are maturing.

Other small GTPases are also involved in signalling to adherens junctions. Recent work from our laboratory and others has shown that the Ras-related GTPase Rap1 is activated at maturing cell-cell contacts (Hogan et al., 2004; Price et al., 2004). This is due to recruitment of C3G, a GEF for Rap1, which binds to the C-terminus of E-cadherin at sites of nascent cell-cell contact (Hogan et al., 2004).

To summarise this section, cadherin engagement is clearly involved in numerous 'outside-in' signalling pathways, and also, 'inside-out' signalling can modulate the integrity of existing cadherin-based junctional complexes. What regulates the availability of binding various partners - the catenins, RTKs, or actin and components of Rho GTPase signalling networks - is intensively studied. p120 catenin is currently the focus of work clarifying the nature of some of the molecular mechanisms involved that lead to cadherin signalling, and this is discussed in the next section.

1.3 THE ROLE OF p120 IN SIGNALLING

1.3.1 p120 catenin

p120 was originally identified as a prominent Src substrate (Reynolds et al., 1989). Following sequence alignments that revealed ten Armadillo repeat motifs that showed some homology to those of β-catenin and plakoglobin, p120 was recategorised as a catenin (Reynolds et al., 1994; Reynolds et al., 1992). As a result of this homology to β-catenin, experiments were conducted to show that p120 could also bind to the intracellular domain of cadherins, although to the juxtamembrane domain (JMD) rather than to the so-called catenin binding domain (CBD) (Finnemann et al., 1997; Lampugnani et al., 1997; Reynolds et al., 1994; Shibamoto et al., 1995; Yap et al., 1998). p120 does not share functions with β-catenin in binding to α-catenin or APC (Daniel and Reynolds, 1995) and for a long time its role at the adherens junction was mysterious (Reynolds, 2006).

There is now a large body of evidence to show that p120 is essential in vertebrates, and fulfils many varied roles in the cell (McCrea and Park, 2006; Reynolds, 2006). This section describes the role of p120 as a 'gatekeeper' to regulate cadherin-based cell-cell adhesion (Section 1.3.2); the ability of cytoplasmic and membrane bound p120 to functionally interact with members of the Rho family of GTPases and its impacts on cell morphology and motility (Section 1.3.3); how these dual roles in sessile versus motile behaviour have implications for p120 in tumour progression (Section 1.3.4); and recent evidence for a nuclear role for p120 (Section 1.3.5). Before describing the functions of p120, a summary is given here of what is known about the p120 protein itself.

p120 is the founding member of a subfamily of Armadillo repeat domain proteins

p120 is an evolutionarily conserved protein, with homologues in *Xenopus*, *Drosophila* and *C. elegans* (Kim et al., 2004; Magie et al., 2002; Pettitt et al., 2003). Invertebrates contain only a single p120 gene, whereas increased diversity exists in vertebrates, especially mammals (McCrea and Park, 2006). The p120 subfamily comprises ARVCF (Armadillo repeat gene deleted in Velo-Cardio-Facial syndrome) (Sirotkin et

al., 1997), δ-catenin/NPRAP/neurojungin (neural plakophilin-related Armadillo protein) (Paffenholz and Franke, 1997; Zhou et al., 1997), and p0071 (Hatzfeld and Nachtsheim, 1996). These proteins share >45% identity with p120 in their Arm domains[2] and, with the possible exception of p0071 (Hatzfeld and Nachtsheim, 1996), bind to and co-localize with classical cadherins at adherens junctions (Lu et al., 1999; Mariner et al., 2000; Paffenholz et al., 1999).

The existence of p120 relatives suggests that they have functional similarities and differences that play out in different cell types. For example, p120 family members may compete for cadherin binding (Lu et al., 1999; Mariner et al., 2000). δ-Catenin/NPRAP is selectively expressed in neuronal tissues, which suggests that it has cell-type specific functions (Paffenholz and Franke, 1997). Thus far, p120 appears to be the most abundant member of the family in most cell types, which suggests it is the dominant player in cadherin function (Anastasiadis and Reynolds, 2000). Moreover, the embryonic lethality of p120 knockout mice suggests that p120 family members cannot compensate for its loss (Reynolds, 2006).

p120 is expressed in multiple isoforms

p120 is expressed in a wide range of tissues, including intestine, brain, uterus, blood vessels, skin, lung, kidney and heart (Golenhofen and Drenckhahn, 2000; Keirsebilck et al., 1998; Montonen et al., 2001), but not in B or T cells, consistent with p120's primary role in cell-cell adhesion (Anastasiadis and Reynolds, 2000). The human p120 gene, *CTNND1*, comprises 21 exons and encodes potentially up to 48 protein isoforms due to many inter- and intra-exonic splicing events (Keirsebilck et al., 1998; van Hengel and van Roy, 2006). Typically, though, four main isoforms are seen in mouse and human (Keirsebilck et al., 1998; van Hengel and van Roy, 2006). Human isoforms, designated 1 to 4, differ from each other in the start codon used (Figure 1.4). Alternative combinations with or without internal exons A-C generate additional isoforms (Aho et al., 1999; Keirsebilck et al., 1998). Several domains have been

[2] There is a second group of p120-related proteins, named plakophilins, which have ~30% identity to p120 in their Arm domains. Unlike the first group, plakophilins localize to desmosomes through interactions mediated by their N-terminal domain. Since the plakophilin subfamily members exhibit higher similarity to one another than p120, and fulfil different functions in the cell, they are designated as a separate subfamily to the p120 subfamily (Anastasiadis and Reynolds, 2000).

identified in p120 (Figure 1.4), including the coiled-coil domain found only in isoform 1, and the phosphorylation domain found in all isoforms except isoform 4 (Keirsebilck et al., 1998; Mo and Reynolds, 1996). Isoform 1A ('long' or 120 kDa) and isoform 3A ('short' or 100 kDa) are expressed predominantly in highly motile fibroblastoid cells and epithelial cells, respectively (Keirsebilck et al., 1998; Mo and Reynolds, 1996). The isoform used in the experiments reported in this thesis is p120-1B.

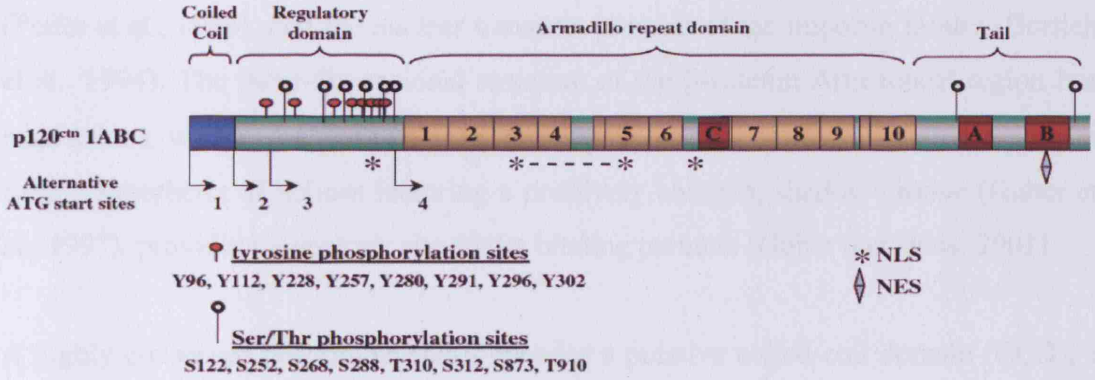


Figure 1.4 p120 isoforms and domain structure. *p120 isoforms 1-4 differ in the ATG start codon used. Exons A, B, and C (red boxes) can be alternatively spliced in or out of the various isoforms. In blue is the coiled coil domain found only in isoform 1. The regulatory domain is also found in the N-terminal region, with red lollipops indicating tyrosine phosphorylation sites, and open lollipops indicating serine/threonine (S/T) phosphorylation sites. The middle section of p120 contains the 10 Arm repeats which are required for cadherin binding. The function of the C-terminus is unknown, but contains two S/T phosphorylation sites. Putative nuclear localisation sequences (NLSs) are shown with asterisks, and a Leptomycin B-sensitive nuclear export sequence (NES) is shown with a pale blue diamond. Schematic from Reynolds and Roczniak-Ferguson, Oncogene, 2004.*

Interestingly, p120 isoform switching can be observed during epithelial to mesenchymal transitions (EMT) in some carcinoma cell lines, with a reduction in the amount of the 100 kDa forms and an increase in the longer isoforms seen in anaplastic thyroid carcinoma cell lines and in prostate carcinoma cell lines (van Hengel and van Roy, 2006). However, the pattern of isoform expression is very heterogeneous in human cancer cell lines (Keirsebilck et al., 1998; Wu et al., 1998) and overall there is no clear correlation between the expression of particular p120 isoforms and cell transformation.

p120 structure

Figure 1.4 shows the domain structure of p120. All isoforms contain the central Arm repeat domain, which is required for cadherin binding (Daniel and Reynolds, 1995). Arm repeats are tandemly repeated sequences containing approximately 45 amino acids, and are found in a number of proteins of diverse function, including β-catenin and plakoglobin, the APC protein that complexes with β-catenin, a RhoGEF smgGDS (Peifer et al., 1994), and the nuclear transport proteins of the importin family (Gorlich et al., 1994). The three-dimensional structure of the β-catenin Arm repeat region has been solved, which revealed that each Arm repeat consists of three helices that pack to form a superhelix of helices featuring a positively charged, shallow groove (Huber et al., 1997), providing a docking site for its binding partners (Huber and Weis, 2001).

A highly conserved N-terminal motif encodes a putative coiled-coil domain (CCD), a motif typically involved in protein-protein interactions (Figure 1.4; (Anastasiadis and Reynolds, 2000). This region is spliced out in p120 isoforms 2, 3, and 4, whose translation begins at downstream ATGs (Keirsebilck et al., 1998). Given that p120 type 1 isoforms are more prevalent in motile cell types (e.g. fibroblasts, as mentioned above), the CCD might be important for promoting more dynamic cadherin interactions. Alternatively, this domain could be responsible for the recruitment of novel effectors to the cadherin complexes (Anastasiadis and Reynolds, 2000).

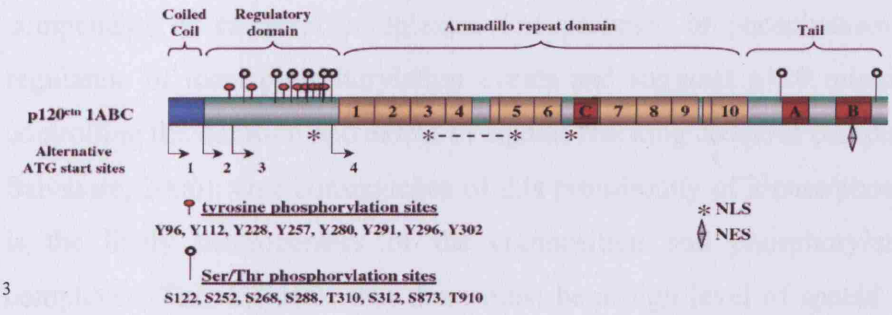
Recent work has identified a number of putative nuclear localisation sequences (NLSs) and a nuclear export sequence (NES) in the p120 sequence (Figure 1.4). A bi-partite NLS is found in the phophorylation domain (Aho et al., 2002). Two studies report the existence of an NLS between Arm repeats 6 and 7 of p120 isoform 1 (Kelly et al., 2004; Mariner et al., 2000). However, in another study, it was found that deletion of this NLS and the putative N-terminal NLS in the phosphorylation domain had no significant effect on nuclear localisation of p120 isoform 3 (Roczniak-Ferguson and Reynolds, 2003). Instead, the Arm repeats 3 and 5 are reported to be necessary and sufficient for nuclear localisation of this isoform of p120 (Roczniak-Ferguson and Reynolds, 2003). This suggests that particular p120 isoforms utilize different pathways for nucleocytoplasmic trafficking.

Observations of cellular localisations of various isoforms of p120 showed that splice variants containing exon B have not been detected in the nucleus (van Hengel et al., 1999). A putative NES with a characteristic leucine-rich motif is encoded by exon B (van Hengel et al., 1999). Experiments in this thesis show that in the presence of Glis2, p120 isoform 1B can be detected in the nucleus (Chapter 4), suggesting that shielding this sequence may impact on the nucleocytoplasmic shuttling of p120.

The N-terminal region of p120 contains a high number of tyrosine, serine, and threonine residues which collectively are known as the phosphorylation, or regulatory, domain (Figure 1.4, repeated below[3]; (Alema and Salvatore, 2006). These residues are present in all isoforms except isoform 4, whose ATG start codon resides N-terminal to the Arm repeat domain (Keirsebilck et al., 1998). However, although isoform 4 can be detected at the mRNA level by real-time-PCR (RT-PCR), it is rarely observed at the protein level and may not be physiologically relevant in cells (Reynolds and Roczniak-Ferguson, 2004).

p120 is highly phosphorylated

The functional consequences of the phosphorylation of p120 are not yet clear. Specific phosphorylated tyrosines (Figure 1.4) presumably serve as docking sites for the recruitment of partners carrying domains that specifically bind phosphotyrosines (e.g. Src-homology 2 (SH2) domains; (Alema and Salvatore, 2006). Under specific circumstances, both *in vitro* and *in vivo*, tyrosine phosphorylation of p120 correlated with an increased affinity for cadherins (Calautti et al., 1998; Kinch et al., 1995; Piedra et al., 2003; Roura et al., 1999). However, other studies show that there is no significant change in p120/E-cadherin association even in instances in which p120 was heavily tyrosine phosphorylated such as in v-Src transformed cells or upon RTK



3

stimulation (Mariner et al., 2004; Ozawa and Ohkubo, 2001; Papkoff, 1997; Reynolds et al., 1994; Shibamoto et al., 1994). These apparently contrasting results most probably reflect a complex and fine regulation of the association of p120 with cadherins. It is possible, for instance, that individual tyrosine phosphorylation sites differentially affect the affinity of p120 for E-cadherin. Additionally, the association of p120 to cadherins may be influenced by concomitant phosphorylation events on other components of the cadherin complexes (Alema and Salvatore, 2006).

Src is a major kinase targeting p120, and indeed was the route to the identification of p120 in the first place (Reynolds et al., 1992). Most prominent Src substrates (for example, tensin, focal adhesion kinase (FAK), Crk-associated substrate, cortactin, ezrin-radixin-moesin proteins) are proteins that affect the organisation of the actin cytoskeleton (Brunton et al., 2004), so it seems likely that Src phosphorylates p120 in situations when p120 can take part in signalling to the cytoskeleton (as described below in Section 1.3.3). The tyrosine phosphorylation of p120 by Src is presumed to lead to the disruption of AJs. But since Src and other Src family kinases (SFKs) target multiple proteins at the AJ, and knockout studies show both positive and negative effects on AJ stability and turnover (Calautti et al., 1998), it is not clear whether modifications of p120 by Src are responsible for effects on AJ regulation (Brunton et al., 2004), and direct mechanistic evidence is lacking (Mariner et al., 2001). Results presented in this thesis show a novel role for Src phosphorylation of p120 in enhancing the interaction between p120 and the transcription factor Glis2, and stimulating the p120-induced C-terminal cleavage of the Glis2 protein (Chapter 3).

p120 can physically associate with the SFKs Fyn and Yes (Piedra et al., 2003), and the tyrosine phosphatases PTPμ (Zondag et al., 2000), DEP1 (Holsinger et al., 2002), and SHP1 (Keilhack et al., 2000; Mariner et al., 2001), suggesting that p120 acts as a scaffold/adaptor site to modulate tyrosine phosphorylation of itself and other components of cadherin complexes. The presence of phosphatases implies a tight regulation of local phosphorylation events and suggests p120 might be involved in controlling the duration and extent of signals reaching cadherin complexes (Alema and Salvatore, 2006). One consequence of this promiscuity of kinase/phosphatase partners is the likely heterogeneity of the composition and phosphorylation patterns of complexes. This indicates that there must be a high level of spatial and/or temporal

control of cadherin at AJs, via phosphorylation events. These data give further weight to the description of p120 as a signal integrator at AJs (Brunton et al., 2004; Reynolds and Roczniak-Ferguson, 2004).

In addition to tyrosine phosphorylation, p120 is phosphorylated on serine and threonine (S/T) residues (Aono et al., 1999; Downing and Reynolds, 1991; Ratcliffe et al., 1997; Ratcliffe et al., 1999; Wong et al., 2000). The majority of these sites are clustered in the phosphorylation domain (Figure 1.4). However, S873 and T910 are located in the C-terminal end indicating a second regulatory region (Xia et al., 2003). Unidentified kinases are responsible for the 'constitutive' phosphorylation of p120 at S/T residues. However, the pharmacological activation of protein kinase C (PKC) selectively promotes the phosphorylation of S873 at the C-terminus, and concomitantly induces partial dephosphorylation of multiple sites at the N-terminus (Xia et al., 2003). The consequences of S/T phosphorylation are still largely unknown, and a recent report using new site- and phospho-specific antibodies to p120 in a variety of cell lines indicates that the S/T phosphorylation status of p120 does not affect its role in cadherin complex stabilisation, despite a requirement for membrane localisation of p120 for its availability as a substrate (Xia et al., 2006).

Cellular localisation of p120

p120 exists in four pools in the cell. It is predominantly found at cell-cell contacts associated with cadherins (Reynolds et al., 1994), but can also be seen to associate with microtubules (Chen et al., 2003; Franz and Ridley, 2004; Yanagisawa et al., 2004), and is subject to nucleocytoplasmic shuttling (van Hengel et al., 1999). These different localisations in the cell reflect the signalling roles of p120 in cell-cell adhesion, trafficking, and regulation of gene transcription, respectively. Furthermore, mislocalisation of p120 is often seen in human tumours (Thoreson and Reynolds, 2002). These roles of p120, and the potential contribution of p120 to tumour progression are discussed below.

1.3.2 p120 and cadherin trafficking

p120 is a central regulator of cadherin turnover (reviewed in (Xiao et al., 2006). By modulating cadherin membrane trafficking and degradation, p120 levels act by a set point mechanism to modulate cell-cell adhesive interactions. The precise molecular mechanism by which this process occurs is still being elucidated, although there is substantial evidence to suggest that p120 binding to cadherin selectively prevents cadherin endocytosis and degradation (Xiao et al., 2006). Stable cell-cell adhesion, therefore, relies on stabilisation of cadherin at the cell surface by p120. A summary of what is known about cadherin regulation by p120 is given here, and a possible model of its action is also shown in Figure 1.5.

Direct evidence that p120 plays a critical role in cadherin stability came from the analysis of SW48 colon carcinoma cells, which have mutations in the p120 gene resulting in very low expression (a heterozygous nonsense mutation which results in the premature truncation of p120 at the third Arm repeat; the other allele had severe cDNA abnormalities resulting in premature stop codons (Ireton et al., 2002). These tumour cells were only loosely organised, and failed to maintain epithelial morphology (Ireton et al., 2002). This phenotype was due to strongly reduced levels of E-cadherin; interestingly, mRNA levels of E-cadherin were unaffected, but E-cadherin stability at the protein level was significantly reduced (Ireton et al., 2002). Reintroduction of p120 or forced high expression of E-cadherin led to a restoration of epithelial morphology (Ireton et al., 2002).

Further confirmation of the importance of p120 in regulating cadherin stability came from parallel studies from the Reynolds and Kowalczyk laboratories, in which siRNA of p120 in a number of cell lines showed a reduction in steady-state cadherin levels and associated loss of cell-cell adhesion (Davis et al., 2003; Xiao et al., 2003). Furthermore, these effects are not specific for E-cadherin – p120 knock-down also destabilised VE-cadherin and N-cadherin (Davis et al., 2003; Xiao et al., 2003). Both groups found that by adding back increasing amounts of p120, the levels of cadherin rose in parallel, suggesting that p120 acts as a 'rheostat' to regulate cadherin levels.

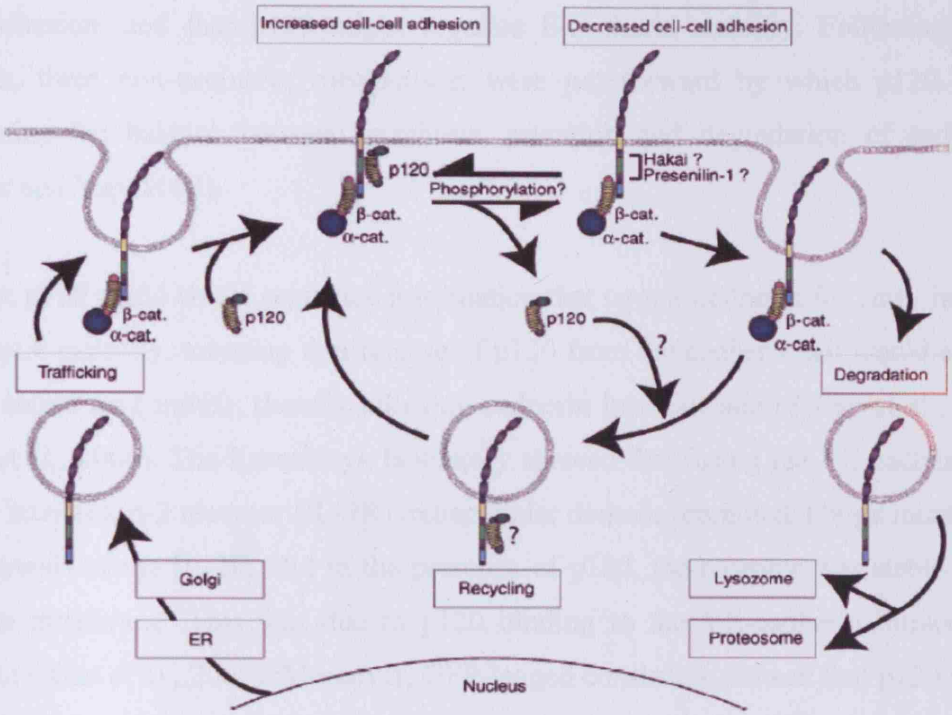


Figure 1.5 Model for p120 function in regulating cadherin turnover. *Cadherin stability seems to be tightly regulated by direct physical binding of p120. Upon arrival at the cell surface, E-cadherin is immediately internalised and degraded if p120 is unavailable. p120 acts as a 'cap' to prevent internalisation of E-cadherin, either by preventing binding of other proteins, such as Hakai or Presenilin-1, which destabilise the cadherin-catenin complex, or by shielding phosphorylation sites/sequence information that recruit components of the internalisation machinery. Whether p120 is involved in recycling of E-cadherin is not known, but association of p120 with cadherin-containing vesicles moving on microtubule tracks has been observed (Chen et al., 2003). Schematic from Reynolds and Carnahan, Sem Cell Dev Biol, 2004.*

Thus, it seems extremely likely that the p120 interaction with the JMD of cadherin regulates cadherin expression levels by controlling its turnover rates. Evidence from other laboratories indicates that in some human tumours, the loss of p120 correlates with the loss of cadherin expression (reviewed in (Thoreson and Reynolds, 2002). Collectively, these data provide evidence that p120 is essential for stable mammalian[4]

[4] Studies in *Drosophila* and *C. elegans* suggest that while p120 is clearly a positive regulator of cadherin-based adhesion, it is not essential, since p120 ablation experiments result in viable organisms (Xiao et al., 2006). The situation is different in vertebrates, where knockout studies clearly indicate a vital role for p120 *in vivo* (Davis and Reynolds, 2006; Perez-Moreno et al., 2006).

cell adhesion, and that p120 might regulate E-cadherin stability. Following these reports, three non-exclusive mechanisms were put forward by which p120 might determine the balance between synthesis, retention and degradation of cadherins (Peifer and Yap, 2003).

Firstly, p120 could shield sequence information that targets cadherin for entry into the endocytic pathway, meaning that release of p120 from the cadherin tail would expose these amino acid motifs, thereby allowing cadherin internalisation (Xiao et al., 2005; Xiao et al., 2006). The Kowalczyk laboratory showed that fusing the VE-cadherin tail to the interleukin-2 receptor (IL-2R) extracellular domain promoted a huge increase in endocytosis of the IL-2R. But in the presence of p120, the receptor was stable at the plasma membrane. This was due to p120 binding to the VE-cadherin intracellular domain (Xiao et al., 2005). Moreover, GFP-tagged constructs showed that p120 would then dissociate from the VE-cadherin tail upon internalisation (Xiao et al., 2005). Interestingly, the VE-cadherin tail contains several putative tyrosine and dileucine internalisation motifs which may mediate endocytosis, and one of these motifs lies near the p120 binding site, further supporting a model in which p120 serves as a 'cap' that prevents clathrin adaptor proteins from interacting with the cadherin intracellular tail (Xiao et al., 2006).

The second of the three mechanisms put forward to explain p120's function in cadherin trafficking involves a more direct role in cell adhesion, since many cadherin mutants which cannot bind p120 are poorly adhesive (Thoreson et al., 2000). This suggests changes in lateral clustering of cadherins or altered cadherin-activated signalling (Goodwin et al., 2003). This is supported by a recent report from the Takai laboratory where the adhesion molecule nectin was found to inhibit E-cadherin endocytosis by stabilising the association of p120 with E-cadherin through the formation of a trimeric complex consisting of afadin, Rap1, and p120 (Hoshino et al., 2005). The stabilisation of the cadherin intracellular tail is also due to binding to β-catenin, as recent NMR data shows that this interaction gives structure to the intracellular domain of cadherin, and this is proposed to protect the cadherin tail from proteolysis (Huber et al., 2001).

Thirdly, p120 may participate in the delivery of cadherin to the cell surface. Cadherin-containing vesicles have been shown to move on microtubule tracks towards cell-cell contacts (Mary et al., 2002), and p120 was recently shown to bind to both kinesin and directly to microtubules (Chen et al., 2003; Franz and Ridley, 2004; Yanagisawa et al., 2004). Studies using timelapse imaging of GFP-p120 showed that p120 was associated with cadherin-containing vesicles moving along microtubules towards cell-cell contacts (Chen et al., 2003). Furthermore, mutants that disrupt the cadherin/p120/kinesin linkage retarded the rate at which cadherins reaccumulated in cell-cell contacts following a calcium switch[5] (Chen et al., 2003).

These findings may also lead to a re-interpretation of previous findings that showed forced expression of one type of cadherin can downregulate expression of endogenous cadherins - for example, the 1996 report from Islam et al. mentioned in Section 1.2.2, which showed that overexpression of N-cadherin can lead to the downregulation of E- and P-cadherins (Islam et al., 1996). If competition for a limited pool of p120 is necessary for stable expression at the plasma membrane, then forced overexpression of an exogenous cadherin would out-compete endogenous cadherins for p120 binding to the JMD, thus leading to endocytosis and degradation of endogenous cadherins (Xiao et al., 2006).

A critical question is how p120 dissociates from the cadherin cytoplasmic tail. Both Presenilin-1 and Hakai can also interact with the JMD of cadherin and are implicated in cadherin internalisation. Hakai is an E3 ligase for E-cadherin (Fujita et al., 2002), and is discussed in Section 1.5. Presenilin-1 promotes E-cadherin degradation by proteolytic cleavage of the cadherin cytoplasmic tail (Baki et al., 2001; Marambaud et al., 2002). These proteins may compete with p120 for binding of the cadherin tail. Indeed, it was shown recently that p120 binding to the JMD of E-cadherin was reduced in the presence of v-Src and overexpressed Hakai (Tricaud et al., 2005). Again, this suggests that p120 may function as a cap to shield the cadherin tail from interactions which would lead to its internalisation and degradation. Signals that cause the

[5] The calcium switch experiment involves depleting extracellular calcium, causing the loss of homophilic Ca^{2+}-dependent interactions between EC repeats of cadherin and the subsequent internalisation of cadherin. Reintroduction of normal calcium levels leads to the re-establishment of cadherin-based cell-cell contacts.

phosphorylation of the E-cadherin tail (which is critical for Hakai binding; (Fujita et al., 2002) would favour Hakai rather than p120 binding. Since VE- and N-cadherin lack the tyrosine residues which are necessary for Hakai binding (Fujita et al., 2002), other Hakai-like molecules may couple selectively to specific cadherins.

Despite this wealth of evidence to show a positive role for p120 in cadherin stability, there have also been reports that show p120 can have a destabilising effect on adhesive activity. For example, in Colo205 cells, E-cadherin function is severely impaired despite the cells having normal levels of E-cadherin and catenins (Aono et al., 1999). Adhesion can be restored by p120 mutants lacking most of the N-terminal region, but not by full-length p120 (Aono et al., 1999). Treatment of these cells with staurosporine, the serine/threonine kinase inhibitor, also induces cadherin-mediated cell aggregation, implicating hyperphosphorylation of p120 in this pathway (Aono et al., 1999). This result implies an aberrant signalling pathway in Colo205 cells that acts constitutively through an N-terminal region of p120 to block E-cadherin function. Thus, full-length p120 appears to negatively regulate adhesion in Colo205 cells. Although clearly exceptional, these data are corroborated by two other reports in K-562 leukaemia cells and murine L-fibroblasts (Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998). Minimal mutation of the JMD (thereby ablating p120 binding) restores adhesive ability of an E-cadherin construct that cannot otherwise promote aggregation in K-562 cells (Ozawa and Kemler, 1998). Inhibition of adhesion again correlated with hyperphosphorylation of p120 in L-cells (Ohkubo and Ozawa, 1999).

Integrating these observations of both positive and negative regulation of adhesion by p120 and the JMD is difficult. One suggestion is that *trans* binding between cadherins is somehow linked to the positive effect of the JMD, whereas intracellular signalling can induce its negative function (Anastasiadis and Reynolds, 2000). Thus, p120 could act as a switch that induces cadherin clustering and strong adhesion when 'activated', and also mediate junction disassembly following signalling events leading to its 'inactivation' (Anastasiadis and Reynolds, 2000). As described in Section 1.3.1, how phosphorylation is involved in p120 activity status is currently unclear.

As briefly referred to above, actin cytoskeleton interactions and signalling downstream of p120 may also affect cadherin stability. These processes include interactions of p120 with the Rho family of small GTPases, and this is discussed in the next section.

1.3.3 p120 and Rho small GTPases

There is now strong evidence to suggest that p120 can act through regulation of Rho GTPases. Rho family GTPases play important roles in the regulation of cytoskeletal organisation and dynamics during cell spreading and migration (Etienne-Manneville and Hall, 2002). Rho can induce the formation of stress fibres, while Rac and Cdc42 promote the formation of lamellipodia and filopodia, respectively (Etienne-Manneville and Hall, 2002). Reports from several laboratories suggest that p120 can inhibit RhoA (Anastasiadis et al., 2000; Noren et al., 2000) and activate Rac and Cdc42 (Grosheva et al., 2001; Noren et al., 2000). Activation of Rho GTPase signalling pathways downstream of p120 have been described during processes that enhance both cell-cell adhesion and cell motility. The main evidence for the p120-Rho connection is given here, with an appraisal of the discrepancies between studies showing that p120 must be free in the cytoplasm to affect Rho signalling, or can functionally interact with Rho GTPases as part of the AJ complex.

The Reynolds laboratory investigated the molecular basis for the classic 'branching' phenotype observed in fibroblastic cells overexpressing p120, and showed that cytoplasmic p120 potently inhibited RhoA (Anastasiadis et al., 2000). Co-expression of dominant active (DA) RhoA completely blocked the effect, while co-expression of dominant negative (DN) RhoA increased the proportion of cells exhibiting extreme branching (Anastasiadis et al., 2000). In addition to RhoA inhibition, p120 can activate Cdc42 and Rac (Noren et al., 2000). In support of these findings, another study showed that overexpressing p120 at a moderate 5-fold increase on normal levels promoted filopodial and lamellipodial activity and augmented migratory ability, while inhibiting contractility and focal adhesions in fibroblasts (Grosheva et al., 2001). Moreover, co-transfection of DA Rho, or DN Cdc42 and DN Rac, suppressed these morphological effects (Grosheva et al., 2001). Interestingly, co-transfection with an

excess of E-cadherin led to sequestration of p120 at cell-cell junctions or small cadherin-containing vesicles, and abolished the effects on cell morphology (Grosheva et al., 2001). This supports experiments in which cells expressing mutant cadherins unable to bind p120 allows the resultant cytoplasmic pool of endogenous p120 to affect Rho signalling (Anastasiadis et al., 2000).

Recent *in vivo* data supports these studies. Using conditional gene targeting in mice, p120 ablation in the epidermis has recently been achieved by the Reynolds and Fuchs laboratories (Perez-Moreno et al., 2006). Pull down assays show increased RhoA activity in p120 null keratinocytes, and this contributed to NFκB activation in these cells (Perez-Moreno et al., 2006). Furthermore, this occurred using primary keratinocytes in low-calcium medium *in vitro*, suggesting that AJ components, including E-cadherin, are not involved in this pathway (Perez-Moreno et al., 2006).

From this work, it seems that p120's ability to shuttle between cadherin-bound and cytoplasmic pools is key to its dual roles in cell-cell adhesion and cell motility. This idea is supported by the weaker interaction between N-cadherin and p120 compared to E-cadherin. N-cadherin is expressed in fibroblastic cell types, which are more highly motile than epithelial cells that express high levels of E-cadherin (Anastasiadis and Reynolds, 2001). This also gives clues to why sparse cultures of cells (low cadherin-based junctions, high cytoplasmic p120) are more motile than denser colonies (high cadherin-based cell-cell contacts, low cytoplasmic p120) (Anastasiadis and Reynolds, 2001).

However, more recent reports challenge this model. An investigation of Rac activation downstream of cadherin ligation showed a role for AJ-associated p120 (Goodwin et al., 2003). A minimal mutation in the JMD of E-cadherin that inhibits p120 binding is no longer able to promote activation of Rac and is associated with profound defects in the establishment of stable adhesive contacts (Goodwin et al., 2003). This suggests that p120 in the cadherin complex can activate Rac. In addition, recent work from the Reynolds lab indicates that p120 can target suppression of Rho to cadherin complexes via transient recruitment of p190RhoGAP (Wildenberg et al., 2006). Further, a study of invasive ability in p120 siRNA knockdown carcinoma cell lines shows that reintroduction of p120 rescues motility and invasiveness of these cells, but a cadherin-

uncoupled p120 mutant cannot (Yanagisawa and Anastasiadis, 2006). This study also shows that Rac1 activation is dependant on p120 competent to bind cadherin (Yanagisawa and Anastasiadis, 2006). How these three reports can be combined satisfactorily with the previous studies is not yet clear. It is important to note at this point that many of these studies were done in CHO (Chinese hamster ovary) cells (Goodwin et al., 2003) or fibroblasts (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000) and the results extrapolated to explain the behaviour of epithelial cells; it is possible that cell type differences may account for differential activities of some of these signalling molecules.

The mechanism of action of p120 in Rho GTPase signalling is still being investigated. A report in *Drosophila* has shown that p120 can bind directly to Rho1 (Magie et al., 2002). Using murine constructs, *in vitro* GDP/GTP exchange assays show that in the presence of purified GST-p120 protein, RhoA, but not Rac1, was inhibited in its intrinsic GDP dissociation activity, suggesting p120 could bind to RhoA in the manner of a RhoGDI, although a direct interaction by yeast 2-hybrid or co-immunoprecipitation could not be shown (Anastasiadis et al., 2000). Another report suggests that the mechanism of action is via the RhoGEF, Vav2 (Noren et al., 2000). A direct interaction between p120 and Vav2 is shown by immunoprecipitation, and a Vav2 deletion mutant was shown to repress p120-induced branching. Vav2 can activate RhoA, RhoG, Cdc42, and Rac1 (Abe et al., 2000; Schuebel et al., 1998), suggesting that the previously observed inhibition of RhoA by p120 (Anastasiadis et al., 2000) must be occurring through some other mechanism, perhaps via p190RhoGAP (Wildenberg et al., 2006). Because it has been reported that Rac and Cdc42 can inhibit RhoA in some cell types (Nimnual et al., 2003; Sander et al., 1999), it is possible that inhibition of RhoA by p120 occurs indirectly through Rac and Cdc42 activation. The GDP/GTP exchange assays described above do not support this indirect activation hypothesis though (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001), but perhaps these two mechanisms are not mutually exclusive, as the p190RhoGAP data shows that DA Rac can cause the activation and recruitment of p190RhoGAP to cell-cell contacts leading to subsequent Rho inhibition (Wildenberg et al., 2006). Further studies that will also address the roles of the various kinases and phosphatases that act on p120 in its cadherin-associated state will hopefully further illuminate the function of p120 in Rho regulation.

Thus, from the evidence presented in this section and the previous section, it is clear that p120 has dual roles in the regulation of both cadherin-based cell-cell adhesion and cell motility. Dependent on the stimulus, it seems that on one hand p120 can promote sessile behaviour in cells, suggesting a potential role as a tumour suppressor, and then in other cases, p120 may also function to promote migratory capacity, indicating that it could be important in metastatic behaviour. The implications of roles for p120 in tumour progression are now considered.

1.3.4 p120 and cancer

p120 can be mislocalised to the cytoplasm in some tumours, or expression can be completely lost (Reynolds and Carnahan, 2004; Thoreson and Reynolds, 2002). As discussed in Section 1.3.2, siRNA-induced downregulation of p120 in cultured cells directly causes degradation of E-cadherin (and associated α- and β- catenins) and can cause almost complete loss of cell-cell adhesion (Davis et al., 2003). Furthermore, p120 ablation *in vivo* causes E-cadherin deficiency and severe defects in adhesion, cell polarity and epithelial morphology in mouse embryonic salivary glands (Davis and Reynolds, 2006). Moreover, these changes closely phenocopy high-grade intraepithelial neoplasias, that in humans progress in a significant proportion of cases to become invasive cancers (Davis and Reynolds, 2006). These observations predict that p120 downregulation in tumours should cause a corresponding loss of members of the cadherin complex. Indeed, a recent prostate cancer study finds p120 downregulation in 70% of high-grade tumours, with a very high incidence of co-downregulation of all components of the cadherin complex (Kallakury et al., 2001).

p120-independent mechanisms of E-cadherin downregulation also occur frequently in human tumours, such as promoter methylation, or mutations or deletions in the gene itself, leading to an accumulation of p120 stranded in the cytoplasm (Reynolds and Carnahan, 2004). The observations discussed in Section 1.3.3 that implicate p120 in the regulation of the Rho family of GTPases have led researchers to postulate a role for cytoplasmic p120 in promoting metastasis in E-cadherin-deficient cells. Indeed, it is known that loss of adhesion by itself does not necessarily translate into increased

motility and invasiveness (Reynolds and Roczniak-Ferguson, 2004). Whereas, the abnormally high cytoplasmic levels of p120 associated with E-cadherin-loss could directly dysregulate multiple events mediated by Rho GTPases. In addition to the well-characterized activities of Rho GTPases in cytoskeletal regulation, these proteins can also signal to the nucleus via pathways linked to the MAP kinases JNK, p38α and p38γ, and as shown by the p120 knockout in mouse epidermis, via NFκB (Perez-Moreno et al., 2006), thereby directly influencing transcriptional events relevant to malignancy and chronic inflammation (Reynolds and Roczniak-Ferguson, 2004). These two roles of p120 as a tumour suppressor and a metastasis promoter are illustrated in Figure 1.6.

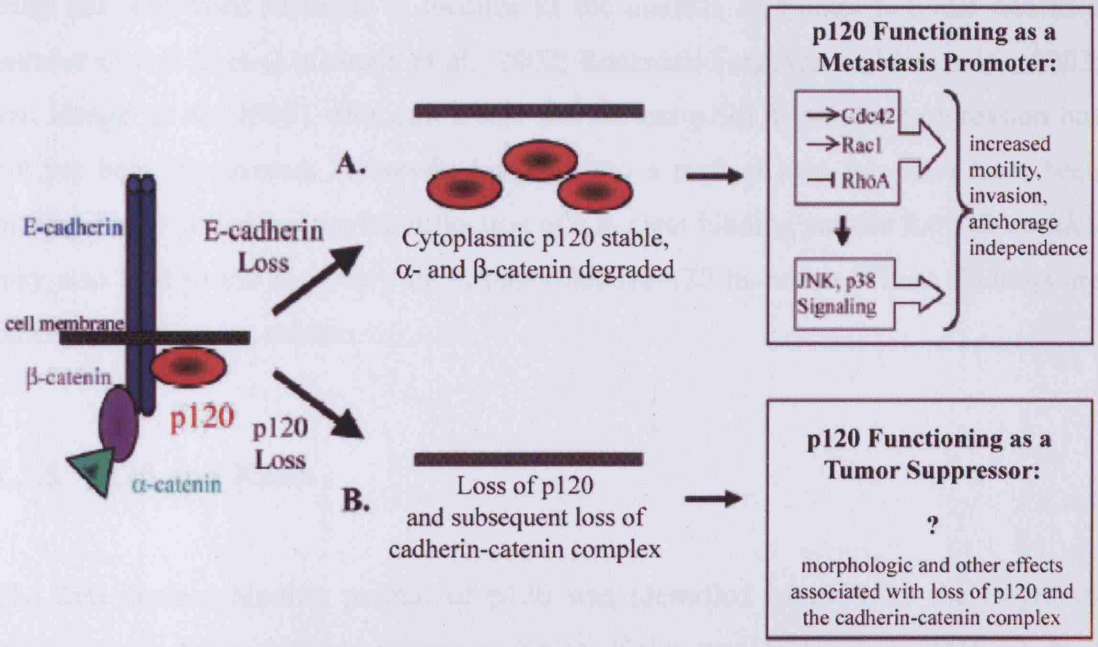


Figure 1.6 Hypothetical roles for p120 as metastasis promoter or tumour suppressor. ***A*** *If E-cadherin loss precedes p120 loss, this leads to degradation of β- and α-catenins, but p120 is stranded in the cytoplasm (and nucleus). p120 could then directly promote metastasis through unregulated effects via Rho GTPases. Rho GTPases also regulate MAPK (JNK, p38) signalling pathways, which are likely to further modulate aspects of the metastatic program.* ***B*** *If p120 loss precedes E-cadherin loss, this leads to destabilization of the cadherin-catenin complex, and has further implications for changes in adhesion, signalling and morphology. Schematic from Thoreson and Reynolds, Differentiation, 2002.*

The recent report from Yanagisawa and Anastasiadis referred to in Section 1.3.3 suggests a possible mechanism for this double role of p120 as promoting both sessile and motile behaviour. They examine the processes involved in epithelial tumour

progression where the loss of E-cadherin expression and inappropriate expression of mesenchymal cadherins coincide with increased invasiveness. Their experiments show that p120 is required for the invasive ability of these tumour cells, by binding to the mesenchymal cadherins' JMDs, thereby stabilising their expression at the plasma membrane and causing concomitant Rac activation (Yanagisawa and Anastasiadis, 2006). In the presence of E-cadherin, however, p120 preferentially binds to the E-cadherin JMD, thereby promoting strong cell-cell adhesion and suppressing invasion (Yanagisawa and Anastasiadis, 2006). Thus it seems that availability of binding partners can affect the outcome of p120 signalling.

p120 has also been reported to localize to the nucleus in human tumours and in a number of cell lines (Lo Muzio et al., 2002; Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999), although a role for nuclear p120 in tumour progression has not yet been discovered. However, insights into a nuclear role for p120 have been made recently following the identification of a nuclear binding partner for p120, which may also lead to the discovery of further roles for 120 in cancer. These findings are described in the next section.

1.3.5 p120 and Kaiso

The first nuclear binding partner of p120 was identified in 1999 by the Reynolds group, as the transcriptional repressor, Kaiso. Kaiso was found in a yeast 2-hybrid screen using full-length p120 as bait, and was characterized as a member of the BTB/POZ (Broad Complex, Tramtrak, Bric a brac/pox virus and zinc finger) domain transcription factor family (Daniel and Reynolds, 1999). Transcription factors of this family are known to play roles in development and cancer progression (Kelly and Daniel, 2006).

Kaiso is localized to the nucleus and can homodimerise (Daniel and Reynolds, 1999). The C-terminus of Kaiso, which includes its three DNA-binding Krüppel-like zinc fingers, binds to Arm repeats 1-7 of p120 (Daniel and Reynolds, 1999). Subsequent studies on Kaiso function showed that it is a DNA methylation dependent transcriptional repressor, binding to methylated CpG islands (Prokhortchouk et al.,

2001), suggesting a role in gene silencing. Kaiso was then also shown to recognise a specific consensus sequence in the promoter of the human and murine *matrilysin* gene (a matrix metalloproteinase) (Daniel et al., 2002). The second and third zinc fingers of Kaiso were shown to be necessary and sufficient for DNA binding (Daniel et al., 2002). In both types of association with DNA, Kaiso recruits co-repressor components to the gene targets (Park et al., 2005; Yoon et al., 2003).

An interaction with a transcription factor provides a strong hint that, like β-catenin, the junctional protein p120 could have nuclear functions in gene regulation. Interestingly, the DNA-binding ability of Kaiso with either of its recognition sites (the consensus sequence or methyl-CpG sites) was inhibited by p120 (Daniel et al., 2002), which supports data showing the interaction between the two proteins shields the zinc finger domains of Kaiso (Daniel and Reynolds, 1999). Further evidence for a nuclear role for p120 with Kaiso was provided when it was shown that mutations in one of the putative nuclear localisation sequences (NLS) of p120 prevented its regulatory effect on Kaiso (Kelly et al., 2004). Using minimal promoter assays, it was shown that the relief of Kaiso-mediated transcriptional repression was dependent on an intact NLS in p120 and nuclear translocation of the p120 protein (Kelly et al., 2004).

Confirmation of the role of p120 in Kaiso-mediated transcriptional regulation came as p120 was shown to inhibit Kaiso-mediated transcriptional repression of the non-canonical Wnt target gene *xWnt11* in *Xenopus* (Kim et al., 2004). This report from the McCrea group showed that after formation of the Kaiso-p120 complex, both proteins dissociate from the *xWnt11* promoter, resulting in gene expression, and this leads to perturbation of gastrulation (Kim et al., 2004).

The p120/Kaiso interaction attracted much attention in 2005, when it was shown that Kaiso could target genes in the canonical Wnt/β-catenin/TCF pathway. This provided evidence for an intriguing convergence of signalling from catenins at the adherens junction on transcriptional regulation of the Wnt pathway. Chromatin immunoprecipitations (ChIPs) and minimal promoter assays showed that p120 could inhibit Kaiso-mediated transcriptional repression of matrilysin, which is a canonical Wnt target gene (Spring et al., 2005). Furthermore, studies in *Xenopus* show that Kaiso

directly represses canonical Wnt gene targets *Siamois*, *c-Fos*, *Cyclin-D1*, and *c-Myc* in conjunction with TCF (Park et al., 2005). In an analogous way to the β-catenin relief of TCF repressive activity, p120 relieves Kaiso-mediated repression of *Siamois*. In addition, Kaiso and TCF co-precipitate in overexpression experiments, and combined Kaiso and TCF derepression results in pronounced *Siamois* expression and increased β-catenin precipitation with the *Siamois* promoter (Park et al., 2005). Functional experiments where β-catenin mRNA was injected into *Xenopus* embryos with Kaiso or an inactive mutant showed that Kaiso (but not the mutant) can suppress β-catenin-induced axis duplication (Park et al., 2005).

However, because either Kaiso depletion or p120 expression in the *Xenopus* embryo, in contrast to β-catenin expression, do not by themselves induce development of the embryonic axis, the canonical Wnt target genes involved in this inductive event are likely to be more strongly regulated by β-catenin/TCF, with a modulatory role for p120/Kaiso (Park et al., 2005). Moreover, there is scant evidence so far that p120 acts downstream of Wnt receptor activation at the cell surface[6]. What signals are involved upstream of p120 and Kaiso in this process are currently unknown. Indeed, whether this coordinate regulation of Wnt gene targets is pertinent to mammalian development is not yet clear, as Kaiso-null mice showed no overt developmental defects (Prokhortchouk et al., 2006), unlike the gastrulation defects seen in Kaiso depleted Xenopus embryos (Kim et al., 2004). It is possible that this is due to functional redundancy in the mouse between Kaiso and other BTB/POZ family members ZBTB4 or ZBTB38 (Filion et al., 2006).

In humans, inappropriate activation of the canonical Wnt signalling pathway is known to be directly associated with tumorigenesis because many Wnt target genes are associated with cell proliferation or invasion (Nusse, 2005). Further, the Kaiso target *matrilysin*, or *MMP-7*, encodes a matrix metalloproteinase directly linked to tumour metastasis through its upregulation in some cancers and proteolysis of the extracellular

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[6] During the preparation of this thesis, Park and colleagues from the McCrea group published a report in Developmental Cell showing that in *Xenopus*, p120 can bind to a functional regulator of Dishevelled, Frodo. p120 is stabilised by this interaction, and upon overexpression of Frodo, an increase in p120-mediated relief of repression of Kaiso targets was observed (Park et al., 2006). A direct link between Wnt activity and Kaiso was not described.

matrix and substrates such as E-cadherin and the β4 integrin (Shiomi and Okada, 2003). Thus, it has been postulated that p120/Kaiso signalling may play a role in cancer progression (Kelly and Daniel, 2006; van Roy and McCrea, 2005). Consistent with this, Kaiso-null mice crossed with tumour-susceptible $Apc^{Min/+}$ mice showed a delayed onset of intestinal tumorigenesis (Prokhortchouk et al., 2006). It is possible to speculate that loss of E-cadherin, as seen in a number of tumour cell lines and cancers (Kallakury et al., 2001; Sarrio et al., 2004; Shibata et al., 2004), could lead to an accumulation of cytosolic p120 which can then enter the nucleus and relieve Kaiso-mediated gene repression. However, nuclear p120 is not seen in many tumour cell lines that have concomitant cadherin loss (van Roy and McCrea, 2005), although nuclear shuttling activities might still be occurring. A histochemical study of Kaiso expression in cancer shows that Kaiso is often lost in the centre of the tumour, and is sometimes found in the cytoplasm rather than the nucleus of those cells still expressing Kaiso (Soubry et al., 2005). The role of Kaiso's methylation dependent gene repression is also important to take into consideration when considering its potential role in human cancer (van Roy and McCrea, 2005). Thus, so far there is no conclusive evidence that p120/Kaiso signalling has a role in tumorigenesis, although many factors suggest it is likely.

A number of important questions about p120 function in the nucleus remain unanswered. What stimulus leads to p120 nuclear translocation is still unknown. Importantly, co-expression of Kaiso and p120 in cells has not been reported to induce the nuclear translocation of p120 (A. Reynolds, personal communication). Work presented in this thesis shows that p120 can bind to another transcription factor, Glis2, and that co-expression of p120 and Glis2 causes the redistribution of p120 into the nucleus of COS-1, HEK293, and L cells (Chapter 4). A summary of what is known about Glis2 is given in the next section.

1.4 GLIS2

1.4.1 Identification of Glis2 as a transcription factor

Glis2 is a Krüppel-like zinc finger protein (Lamar et al., 2001; Zhang and Jetten, 2001; Zhang et al., 2002). Zinc finger proteins constitute one of the biggest families of transcription factors (Klug and Schwabe, 1995). In proteins containing zinc finger motifs, the cysteine and/or histidine metal ligands within the motif fold in a tetrahedral configuration around a central zinc ion (Klug and Schwabe, 1995). The zinc finger motif forms an independent domain containing a two-stranded β-sheet and a short α-helix. Amino acids at three key positions on the surface of this helix play a dominant role in base recognition, conferring the ability of zinc finger domain-containing proteins to recognise specific DNA sequences (Klug and Schwabe, 1995). Members of the Krüppel-like zinc finger family contain two or more Cys_2Hys_2-type zinc fingers that are separated by a conserved sequence (Dang et al., 2000). Proteins in this family can act as activators and/or repressors of gene transcription, and regulate embryonic development as well as a variety of physiological processes in the adult (Dang et al., 2000), and are also implicated in a number of diseases (Turner and Crossley, 1999; Villavicencio et al., 2000).

Glis2 contains five tandem Cys_2Hys_2-type zinc finger motifs, which exhibit the highest homology to those of members of the GLI and Zic subfamilies of Krüppel-like proteins[7] (Figure 1.7; (Zhang and Jetten, 2001). However, Glis2 constitutes a distinct subfamily of Krüppel-like zinc finger proteins as there are differences in locations of splice junctions, primary sequence and spacing of the cysteines and histidines in the zinc finger domain, and the absence of several small highly conserved regions outside the zinc finger domain of GLI proteins (Lamar et al., 2001; Zhang and Jetten, 2001; Zhang et al., 2002). Glis2 was identified simultaneously by two groups, and so is also referred to as NKL, for Neuronal Krüppel Like (Lamar et al., 2001).

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[7] Gli and Glis are confusingly similar names. To emphasize the difference in this thesis, Gli proteins are referred to as GLI.

Analysis of a series of deletion mutants of Glis2 identified an activation domain just upstream of the zinc finger domain, although two adjacent repressor functions further downstream in the sequence (overlapping with the first zinc finger repeat) completely suppress transcriptional activation (Zhang et al., 2002). Results from a one-hybrid analysis of repression of basal transcriptional activation by Glis2 show that Glis2 functions as an active repressor (Zhang et al., 2002). Active repression involves binding to DNA and recruitment of specific co-repressors that mediate the interaction of the repressor with proteins of the basic transcription machinery, as opposed to passive repression where competition for a common DNA binding site or co-activator occurs (Ma, 2005).

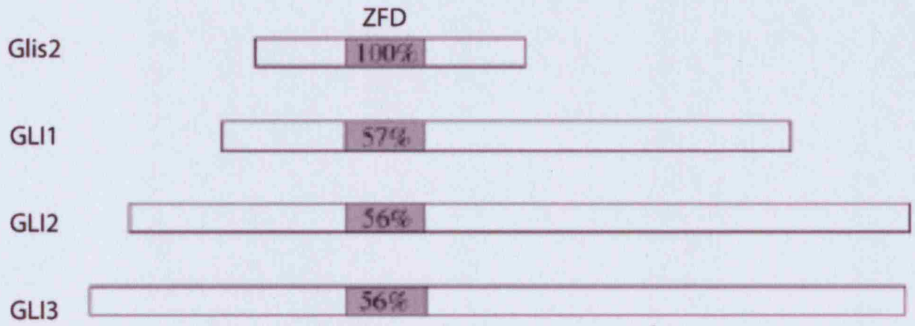

Figure 1.7 Similarity of Glis2 to the GLI family of transcription factors. *The percent identity of the various zinc finger domains (ZFD) of the mouse GLI family with that of Glis2 is indicated. There is little homology in other regions, and variations in the splice junctions indicates that Glis2 forms a separate subfamily member of the zinc finger family of Krüppel-like transcription factors. Schematic from Zhang et al., J Biol Chem, 2002.*

1.4.2 The Glis Family

Three members of the Glis (for GLI similar) family have been identified (Kim et al., 2002; Kim et al., 2003; Zhang and Jetten, 2001). Glis1 and Glis3 share high sequence identity and are thought to be more closely related to each other than Glis2 (Kim et al., 2003). All three proteins have been shown to contain both activator and repressor functions (Kim et al., 2002; Kim et al., 2003; Zhang et al., 2002). During embryonic development, Glis1-3 are expressed in a spatially and temporally regulated way, suggesting roles in the regulation of different steps in organogenesis (Kim et al., 2003;

Lamar et al., 2001; Nakashima et al., 2002; Zhang et al., 2002). Recently, a role for Glis1 was reported in inflammatory epidermis in the skin upon treatment with the tumour promoter PMA (phorbol-12-myristate-13-acetate) (Nakanishi et al., 2006), and a rare neonatal diabetes syndrome associated with congenital hypothyroidism was shown to be due to mutations in the *GLIS3* gene (Senee et al., 2006). There have been no reports on a role for Glis2 in disease states.

1.4.3 Similarity of Glis2 to the GLI/Ci family

The zinc finger domains of Glis2 show homology to the GLI family of Krüppel like transcription factors. The GLI family consists of three members (GLI1-3) and is related to Cubitus interruptus (Ci) in the fly (Osterlund and Kogerman, 2006). Ci and GLI proteins function as downstream regulators of transcription in the Hedgehog (Hh) or Sonic hedgehog (Shh) signal transduction pathways in *Drosophila* and vertebrates, respectively (Osterlund and Kogerman, 2006). GLI proteins can function as activators and repressors of gene transcription and play multiple roles in the regulation of prenatal mammalian development (Ruiz i Altaba, 1999). Further, GLI genes have been implicated in several human diseases, including birth defects and cancer (Ruiz i Altaba et al., 2002). GLI1 is overexpressed in most basal carcinomas of the skin and recent studies have shown that the Shh/GLI pathway is highly activated in small cell lung carcinomas (Villavicencio et al., 2000).

Hedgehog proteins are secreted morphogens and have central roles in embryonic development (Osterlund and Kogerman, 2006). In ventral regions of the central nervous system, Shh controls the specification of progenitor cell fate and neuronal subtype identity (Briscoe and Ericson, 2001; Jessell, 2000). Shh acts as a graded signal, and differential expression of genes downstream of GLI/Ci coordinates which cell types develop (Figure 1.8; (Briscoe and Ericson, 2001). Activation of GLI/Ci involves proteolytic cleavage and translocation to the nucleus, a process that is regulated by interactions with Fused and Suppressor of Fused (SuFu) (Osterlund and Kogerman, 2006). Figure 1.9 summarizes what is known about GLI signalling downstream of Shh.

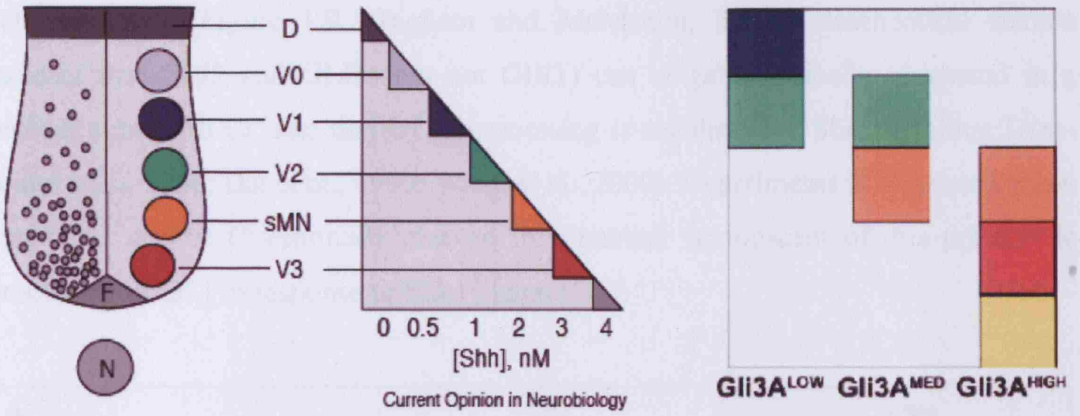

Figure 1.8 Generation of neuronal diversity in response to graded Shh signalling. ***Left panel:*** *Shh is expressed by cells of the notochord (N) and floor plate (F) and is responsible for specifying the subtype identity of neurons generated in the ventral half of the neural tube. V0, V1 and V2 neurons, somatic motor neurons (sMN), and V3 neurons are generated at distinct dorsal-ventral positions in the ventral spinal cord. Shh acts as a graded signal inducing these neuronal subtypes at different concentration thresholds.* ***Right panel:*** *A gradient of GLI transcriptional activity emulates graded Shh signalling and is sufficient to orchestrate patterning of the ventral neural tube. Schematics from Briscoe and Ericson, Curr Op Neurobiology, 2001 (Left panel) and Stamataki et al., Genes & Dev, 2005 (Right panel).*

The molecular mechanisms of Hedgehog signalling are best understood in *Drosophila*, where Ci is the downstream transcription factor. This pathway is the subject of a number of recent reviews (Hooper and Scott, 2005; Osterlund and Kogerman, 2006), and is summarized here (Figure 1.9). In the absence of Hh signalling, Ci is proteolytically processed into a truncated repressor form that inhibits Hh target genes. The processing of Ci is inhibited by Hh signalling, which converts Ci into a transcriptional activator. Hh binds to its receptor Patched, which leads to derepression of another transmembrane receptor Smoothened, and signalling can proceed to a cytosolic complex, the Hedgehog signalling complex (HSC). The HSC consists of Ci, Fused, SuFu, and a number of kinases. Full-length Ci is sequestered in the cytosol by the HSC and its proteolytic processing is mediated by phosphorylation. This cleaved form of Ci can enter the nucleus and inhibit the expression of target genes. Hh signalling causes the release of the kinases from HSC and full-length Ci is free to enter

the nucleus and act as a transcriptional activator. In vertebrates, the process is more complex as the three GLI proteins seem to fulfil various aspects of Ci function between them (Figure 1.9; (Ingham and McMahon, 2001). Biochemical studies indicate that GLI2 and GLI3 (but not GLI1) can be proteolytically processed in a manner similar to Ci, and that GLI3 processing is regulated by Shh signalling (Aza-Blanc et al., 2000; Dai et al., 1999; Wang et al., 2000). Experiments in this thesis show that Glis2 can be C-terminally cleaved in a manner reminiscent of this proteolytic processing of GLI in response to Shh (Chapter 3).

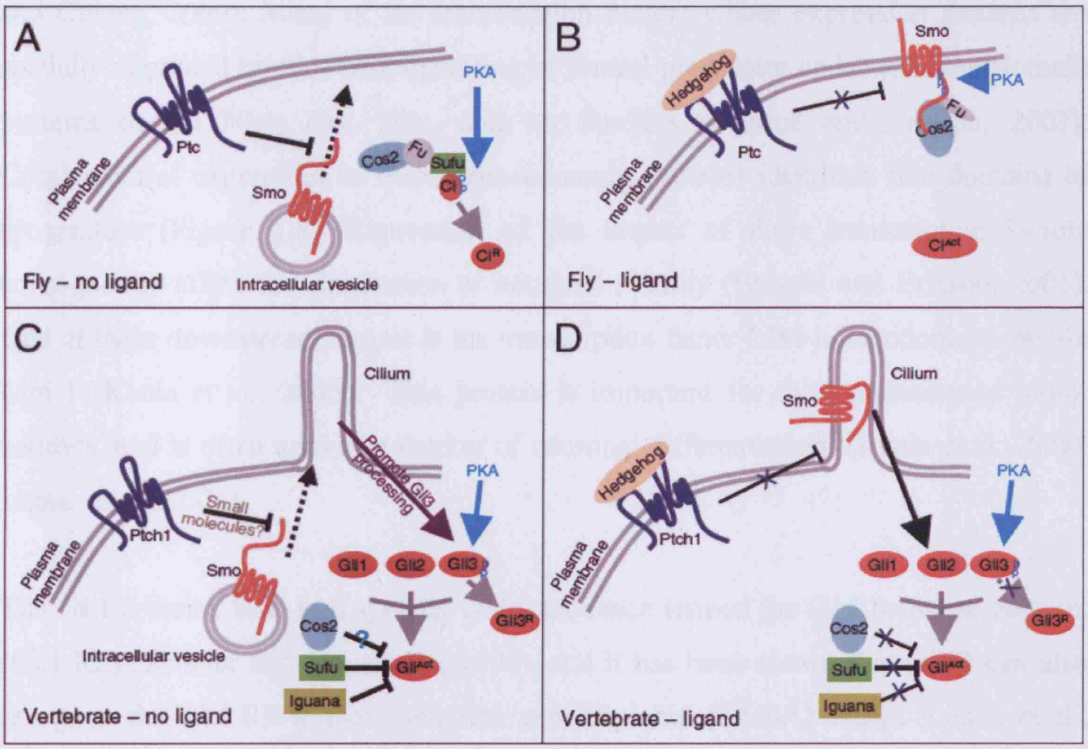

Figure 1.9 GLI/Ci signalling downstream of Shh/Hh. *The Hedgehog pathway in Drosophila and vertebrates are broadly similar, although the presence of three GLI proteins means the process is more complex in vertebrates.* ***A.*** *Drosophila, no Hh present. Patched (Ptc) prevents cell-surface expression of Smoothened (Smo) and Ci forms a complex with Cos2, Fu and SuFu, which targets Ci for proteolytic processing into the repressor form (Ci^R).* ***B.*** *In the presence of Hh, Ptc inhibition is relieved; Smo accumulates at the plasma membrane, forming a complex with Cos2 and Fu and Ci is activated (Ci^{Act}).* ***C.*** *Vertebrates, no Shh present. Ptch1 prevents the accumulation of Smo in the cilium. GLI3 is processed into a repressor form ($GLI3^R$) and the activation of all GLI proteins is inhibited by SuFu (and Iguana in zebrafish) and probably Cos2.* ***D.*** *In the presence of Shh, Ptch1 inhibition is relieved, Smo is targeted to cilia and activates GLI proteins. GLI3 processing is inhibited. Protein Kinase A (PKA) is involved in phosphorylation of Ci and GLI3. Schematic from Huangfu and Anderson, Development, 2006.*

Studies in mice have shown that Shh signalling in the ventral neural tube is transduced by GLI proteins (Aza-Blanc et al., 2000; Ding et al., 1998; Lee et al., 1997; Matise et al., 1998; Park et al., 2000; Ruiz i Altaba, 1998). Embryos lacking GLI2 have defects in the most ventral regions of the neural tube (Ding et al., 1998; Matise et al., 1998; Park et al., 2000), while gain-of-function experiments suggest that Shh signalling acts to repress an inhibitory activity of GLI3 (Aza-Blanc et al., 2000; Lee et al., 1997; Ruiz i Altaba, 1998). This is supported by the observation that in mice lacking both Shh and GLI3, development of motor neurons and ventral interneurons is rescued (Litingtung and Chiang, 2000). Some of the transcription factors whose expression patterns are spatially regulated by Shh/GLI signalling in ventral progenitor cells are homeodomain proteins of the Nkx, Pax, Dbx, and Irx families (Briscoe and Ericson, 2001). Combinatorial expression of these homeodomain proteins identifies five domains of progenitors (Figure 1.8). Expression of the targets of these transcription factors consequently allow the elaboration of neuronal identity (Briscoe and Ericson, 2001). One of these downstream targets is the transcription factor LIM-homeodomain protein Lim 1 (Kania et al., 2000). This protein is important for the maturation of motor neurons, and is often used as a marker of neuronal differentiation[8] (Kania et al., 2000; Lamar et al., 2001).

The GLI proteins bind to a specific DNA sequence termed the GLI response element (GLI RE) (Kinzler and Vogelstein, 1990), and it has been shown that Glis2 can also recognise the GLI RE in electrophoretic mobility shift (EMSA) assays (Lamar et al., 2001). However, no role in Shh signalling has yet been shown for Glis2.

1.4.4 Expression pattern of Glis2

In the developing mouse embryo, Glis2 is expressed from E9.5 in cranial ganglia, the dorsal root ganglia, and neural tube (Lamar et al., 2001). At E10.5 Glis2 is expressed

[8] In the studies reported in this thesis, overexpression experiments in chick neural tube were analysed using antibodies to Pax 6 and Nkx 2.2 as markers of patterning, and Lim 1/2 as a marker of neuronal differentiation (Chapter 4).

in the intermediate zone of the hindbrain and spinal cord. By E12.5 Glis2 expression is restricted to cells in the ventricular zone (Lamar et al., 2001). This is similar in the chick, where the Glis2 homologue is seen in the spinal cord at stage 25 (Lamar et al., 2001). In adult mouse tissues, Glis2 mRNA was most highly expressed in kidney, with low expression in several other tissues including heart, lung, colon, prostate, and brain (Zhang et al., 2002).

1.4.5 Glis2 has a role in neurogenesis

Lamar and colleagues have reported that Glis2 has a role in neurogenesis in the chick neural tube and *Xenopus* embryo. Induction of neural differentiation by injection of transcription factor *Xenopus* Neurogenin (*XNgn1*) mRNA causes ectopic expression of Glis2 outside the neural tube, raising the possibility that Glis2 functions downstream of *XNgn1* to regulate neurogenesis (Lamar et al., 2001). They then show that ectopic *Xenopus* Glis2 expression in the spinal cord promotes premature differentiation of chick neural precursors (Lamar et al., 2001). The molecular mechanism behind the action of Glis2 in this system was not investigated, although suggestions were made that Glis2 could be part of the downstream transcription factor cascade activated in determined precursors, since it is induced by *XNgn1* in a similar way to other neuronal differentiation proteins NeuroD, MyT1 and Xath3 (Lamar et al., 2001). There have been no further reports on Glis2 function in the neural tube since this initial study. The data presented in Chapter 4 of this thesis provides evidence to suggest that the role of Glis2 in neuronal differentiation in the chick neural tube may be inhibitory rather than activating. *Xenopus* Glis2 has 67% identity with mouse Glis2 (Lamar et al., 2001), which is the species of the Glis2 construct used in our study, perhaps indicating species differences between the actions of this transcription factor.

1.5 THE ROLE OF HAKAI IN SIGNALLING

The second part of this thesis describes a preliminary study into novel binding partners of Hakai, from a yeast 2-hybrid study. A description of what is known so far about Hakai and an explanation of why it is a likely candidate for further roles in signalling pathways is set out below.

1.5.1 Hakai is an E3 ubiquitin ligase for E-cadherin

Hakai was identified as a binding partner for tyrosine phosphorylated E-cadherin (Fujita et al., 2002). Hakai is a RING-finger type E3 ubiquitin ligase and binds to E-cadherin upon activation of the tyrosine kinase Src, leading to the ubiquitination and internalisation of the cadherin-catenin complex (Figure 1.10; (Fujita et al., 2002). Subsequently it was shown that internalised E-cadherin is degraded by the lysosome (Palacios et al., 2005). This mechanism is likely to be employed during physiological and pathological processes where cell-cell adhesion is downregulated: during mitosis, or upon the induction of motility and differentiation during EMT (epithelial-mesenchymal transition) in gastrulation or tumour progression. Indeed, in addition to Src, activation of RTKs in epithelial cells, such as the hepatocyte growth factor (HGF) receptor c-Met, or the EGF receptor, leads to tyrosine phosphorylation of E-cadherin and induces cell scattering and a fibroblast-like morphology (Behrens et al., 1993; Stoker and Gherardi, 1991; Weidner et al., 1990). Loss of plasma membrane E-cadherin can also be mediated by the repression of E-cadherin expression by transcription factors, such as Snail/Slug (Batlle et al., 2000; Bolos et al., 2003). Excessive internalisation and degradation of E-cadherin observed when Hakai is overexpressed raises the possibility that Hakai may participate in tumour progression and metastasis (Fujita et al., 2002). A recent paper reports the finding that the MDM2 E3 ligase, originally identified as targeting p53 (Haupt et al., 1997), can also target E-cadherin for ubiquitination and internalisation, and metastatic tumour specimens show an inverse relationship between MDM2 expression and E-cadherin expression (Yang et al., 2005). It would be interesting to also examine such tumour specimens for aberrant Hakai expression.

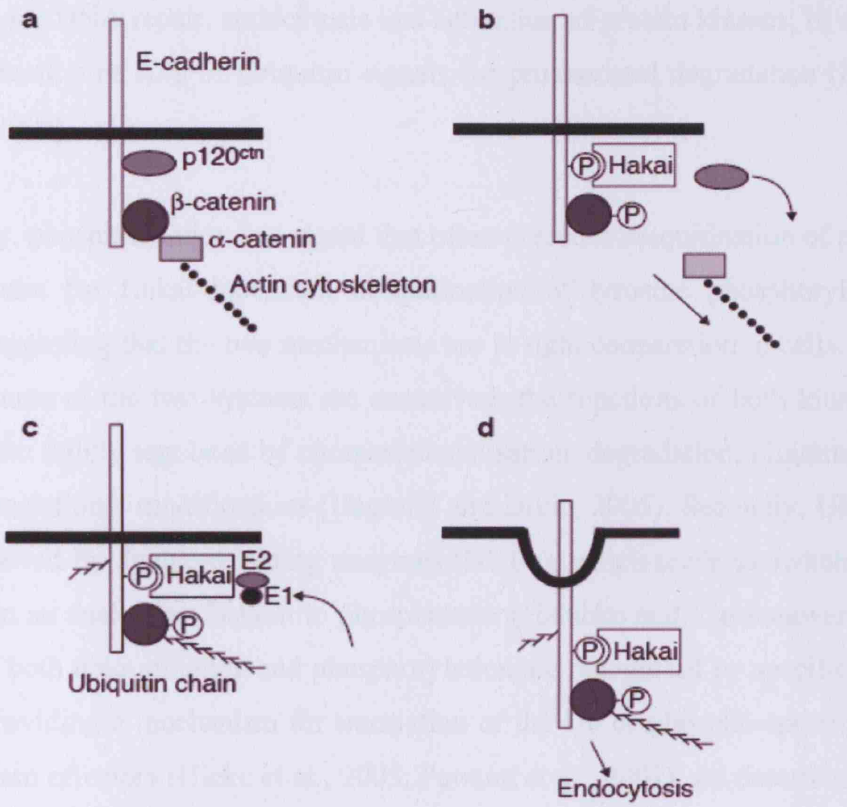

Figure 1.10 Hakai is an E3 ubiquitin ligase for E-cadherin. A. *The E-cadherin cell adhesion system under steady conditions.* ***B.*** *Activation of tyrosine kinases or upregulation of Hakai facilitates the interaction between E-cadherin and Hakai. Tyrosine phosphorylation of E-cadherin favours Hakai binding over p120.* ***C.*** *Hakai mediates ubiquitination of the E-cadherin complex, recruiting E1 and E2.* ***D.*** *The E-cadherin complex is endocytosed. Schematic from Fujita et al., Nature Cell Biol, 2002.*

1.5.2 Ubiquitination and phosphorylation as signalling moieties

The ubiquitin conjugation system is composed of ubiquitin (Ub), the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-ligases (E3). These enzymes function in concert to covalently attach one or more ubiquitin molecules to target proteins after phosphorylation (reviewed in (Hershko and Ciechanover, 1998). Different patterns of ubiquitination can lead to different signalling outcomes in the cell. For example, monoubiquitination has been shown to play a signalling role in endocytosis, endosomal sorting, histone regulation, DNA repair,

virus budding, and nuclear export; polyubiquitination of Lysine 63 (K63) of ubiquitin also signals for DNA repair, endocytosis and activation of protein kinases; in contrast, polyubiquitination of K48 of ubiquitin signals for proteasomal degradation (Haglund and Dikic, 2005).

Interestingly, phosphorylation is a signal that often precedes ubiquitination of proteins, as is the case for Hakai-dependent ubiquitination of tyrosine phosphorylated E-cadherin, suggesting that the two mechanisms are in tight cooperation in cells. Indeed, several features of the two systems are conserved: the functions of both kinases and Ub ligases are tightly regulated by compartmentalisation, degradation, oligomerisation and post-translational modifications (Haglund and Dikic, 2005). Secondly, Ub can be rapidly removed by deubiquitinating enzymes (DUBs), which serve to switch off the Ub signal, in an analogous fashion to phosphatases (Hershko and Ciechanover, 1998). In addition, both ubiquitination and phosphorylation are recognised by specific protein domains, providing a mechanism for translation of the Ub or phospho-specific signal to downstream effectors (Hicke et al., 2005; Pawson et al., 2001). As described above, a difference between the two systems is the variety of types of Ub modifications that are possible. What regulates whether monoubiquitination or polyubiquitination takes place and which lysine residue of ubiquitin is conjugated, is not well understood.

The role of ubiquitin in cadherin-based signalling is so far constrained to cadherin endocytosis, and β-catenin degradation after phosphorylation by GSK3β in the APC scaffold complex (Fujita et al., 2002; Kitagawa et al., 1999). However, as referred to above, Ub can signal to a variety of cellular processes and whether it has further roles to play downstream of cadherin is yet to be discovered. Particularly, whether Hakai has a role to play in further ubiquitination events downstream of cadherin is of interest.

1.5.3 Hakai is ubiquitously expressed suggesting it has other functions

Investigating the possibility that Hakai has other targets in the cell could yield information about the integration of cadherin-based signalling events with other processes. For example, Cbl, a related RING finger domain E3 ligase, can ubiquitinate the EGF receptor, promoting its internalisation and degradation, in two ways. First,

Cbl directly ubiquitinates the EGFR (Waterman et al., 1999). Secondly, the subsequent tyrosine phosphorylation of Cbl by the EGFR leads to recruitment of CIN85, an adaptor protein which is then also ubiquitinated by Cbl (Soubeyran et al., 2002). Protein-protein recognition motifs are thereby created on both the EGFR and CIN85, leading to the recruitment of endophilin and endocytic proteins, which target the complex for lysosomal degradation (Dikic et al., 2003). It is possible that Hakai may also perform a number of functions in order to promote the lysosomal targeting of E-cadherin. In addition, Hakai may have a number of other targets in the cell, since this is often the case for E3 ligases: the Cbl family has over 40 known interaction partners (Dikic et al., 2003). A further impetus to study Hakai function comes from the observation that Hakai is expressed in a wide number of tissues, and can be localised to the nucleus, suggesting that E-cadherin is not its only target (Y. Fujita, unpublished data).

1.6 AIMS OF THE PROJECT

Signalling pathways downstream of cadherin can be transduced through the cytosolic binding partners of cadherin. The well-characterized role for β-catenin in growth control and transcription downstream of Wnt is a classic example. p120 catenin has been shown to be an important regulator of cadherin function, and its presence in the nucleus in various experimental conditions and pathological tissue specimens suggest further signalling roles inside the cell.

The identification of the transcription factor Glis2 as a potential binding partner for p120 was the starting point for an investigation into the function of this interaction. The main aim of this study was to characterize the interaction between p120 and Glis2 and to investigate the function of this complex in the nucleus. Chapter 3 describes the verification of the interaction between p120 and Glis2, and describes the C-terminal cleavage of Glis2 in the presence of p120 and Src. Chapter 4 describes the cellular localisation of p120 in the presence of Glis2, and *in vitro* DNA-binding studies show that both full-length and cleaved Glis2 are able to bind DNA. Further, Chapter 4 contains the findings of Glis2 and p120 overexpression studies in the chick neural tube.

A second binding partner of E-cadherin, Hakai, was also examined. A yeast 2-hybrid assay yielded several clones for investigation and thus the second aim of this study was to verify the authenticity of these potential binding partners. Chapter 5 describes the cloning and expression of these proteins, and reports preliminary data on these interactions.

CHAPTER 2

MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY

2.1.1 Polymerase Chain Reaction

DNA was amplified in a thermocycler (Jencons-PLS). PCR reactions used a final volume of 100 µl containing 20 ng template DNA, 0.2 mM dNTPs (Amersham), 1 µl Vent polymerase (New England Biolabs), 10 pmol of each oligonucleotide primer (MWG Biotech) and 10 µl Vent polymerase 10x buffer (NEB). DNA was amplified using reaction specific programmes within the following parameters: 1x at 95°C for 5 min, 30-35x [95°C for 1 min, 55-65°C for 1 min, 72°C for 2 to 3 min] and 1x 72°C for 10 min. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturers instructions.

2.1.2 Restriction digests

Plasmid DNA and PCR products to be digested (2-5 µg DNA per reaction) were added to 2 µl appropriate restriction enzyme buffer (NEB), 2 µl of 10x Bovine Serum Albumin (BSA, NEB), and 0.6 µl (approximately 10 units) of each restriction enzyme (NEB). Reactions were made up to a final volume of 20 µl using distilled H_2O (dH_2O) and were incubated for 1 hr at either 25°C or 37°C as appropriate. Cut vectors were then further treated with Calf Intestine Phosphatase (CIP, NEB). 160 µl H_2O, 20µl CIP Buffer (NEB), and 4 µl CIP were added to the vector restriction digest mixture, and incubated at 37°C for 1 h. 1 µl of 0.5 M EDTA was then added to the mixture and the reaction was brought to 75°C for 10 min.

2.1.3 Agarose gel electrophoresis

DNA was separated by agarose gel electrophoresis at 130 mA for 1 hr on 1% TAE (Tris-acetate 50x stock: 2 M Tris Acetate 100 mM Na_2EDTA (National Diagnostics), diluted with dH_2O) agarose gels. Ethidium bromide was added to the gel to a final concentration of 0.5 µg/ml to allow visualisation of DNA under UV light. This enabled the integrity and purity of the DNA to be assessed. Samples for electrophoresis were added to an appropriate volume of DNA loading buffer (6x stock: 0.25% Xylene cyanole (w/v), 0.25% Bromophenol blue (w/v), 40% sucrose (w/v) made up in dH_2O). DNA fragment sizes were estimated by comparison with a 1Kb DNA ladder (Invitrogen).

2.1.4 Extraction of DNA from agarose gels

DNA was purified from agarose gels using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturers instructions.

2.1.5 Ligations

Ligation reactions were carried out using a molar ratio of vector:insert of 1:5. Reactions were carried out in a final volume of 20 µl containing 2 µl 10x DNA ligase buffer (Roche) and 1 µl T4 DNA ligase (5 U/µl, Roche). Ligations were incubated overnight at 16°C. Inserts which were ligated into vectors that had been cut with different enzymes were first blunt-ended. 1µl T4 DNA Polymerase (Roche) and 2µl dNTP were added to the restriction digest mixture, and incubated at 37°C for 30 min. The reaction mixture was then brought to 75°C for 10 min, and ligation reactions were carried out as described above.

2.1.6 Preparation of $CaCl_2$ competent *Escherichia coli*

DH5α or BL21 *E. coli* were streaked on an LB agar plate and grown overnight at 37°C. A single colony was picked and used to inoculate a 3 ml starter culture of L-Broth (Luria-Bertani medium: 1% bacto-tryptone (DIFCO laboratories), 0.5% bacto-yeast extract (DIFCO laboratories), 1% sodium chloride made up in de-ionised water). This culture was grown overnight at 37°C with agitation and was then used to inoculate 250 ml of SOB (2% bacto-tryptone (w/v), 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 6.7 – 7.0). This culture was grown at 18°C with agitation until an OD_{600} reading of approximately 0.6 was obtained. The culture was placed on ice for 10 min then the cells were harvested by centrifugation at 4°C for 10 min at 2500 x g. The cells were resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7) and were placed on ice for 10 min. They were then harvested by centrifugation at 4°C for 10 min at 2500 x g. Cells were then gently resuspended in 20 ml ice-cold TB. Dimethyl sulfoxide (DMSO, Sigma) was added to a final concentration of 7% and cells were placed on ice for a further 10 min. Aliquots (200 µl) were snap frozen in liquid nitrogen and stored at -80°C. Competency was tested using the pBluescript vector.

2.1.7 Transformation of *E.coli* by 'heat shock'

Competent cells (DH5α or BL21 strains) were thawed quickly at room temperature and placed on ice. DNA constructs (1 µl of plasmids for amplification, 5 µl of ligation mixtures) to be transformed were placed in 1.5 ml microfuge tubes and mixed with 50 µl competent cell suspension. Tubes were tapped and placed on ice for 5 min. Cells were 'heat shocked' by incubation at 42°C for 45 sec and were then immediately returned to ice for 5 min. Pre-warmed L-Broth (200 µl per transformation) was added and the cells were incubated with moderate agitation for 10 min (amplification) or 30 min (ligation). 200 µl of transformed bacteria were plated onto selective LB agar plates containing either ampicillin (50 µg/ml) or kanamycin (30 µg/ml) as required. Plates were incubated overnight at 37°C.

2.1.8 Bacterial DNA mini preps

Individual bacterial colonies were picked and grown overnight with agitation at 37°C in 3 ml cultures of LB containing appropriate antibiotics. The bacteria were harvested by centrifugation at 5000 x g for 30 sec. The supernatants were removed and the pellets were resuspended in 400 µl TENS buffer (1 mM Tris/Cl pH 7.5, 1 mM EDTA pH 8, 0.1N NaOH, 0.5% SDS). Tubes were inverted and kept at RT for 3 min. 200 µl of 3M NaOAc, pH 5.2 was added to each tube and preparations were mixed thoroughly. Following centrifugation at 5000 x g for 10 min supernatants were transferred to clean tubes. Ice-cold isopropanol (600 µl per reaction) (BDH laboratory supplies) was added and the preparations were vortexed and centrifuged at 5000 x g for 10 min. The supernatant was removed and the pellets were rinsed carefully in 70% ethanol. The pellets were left to air dry for 10 min. The DNA pellets were resuspended in 40 µl dH_2O with 10 µg RNase A per reaction and incubated at 37°C for 5 min. 5 µl of each preparation was examined by restriction analysis.

2.1.9 Bacterial DNA maxi preps

DNA was prepared using GenElute HP Plasmid Maxiprep Kit ® (Sigma) according to the manufacturers instructions. Elution of DNA was followed by an Ethanol precipitation. 500 µl of 3M NaOAc, pH 5.2 and 12.5 ml of 100% ice-cold EtOH were added to the 5 ml eluate. Tubes were centrifuged at 3400 x g for 30 min at 4°C. Supernatants were discarded and pellets were resuspended in 1 ml 70% ice-cold EtOH.

After centrifugation at 5000 x g for 10 min, the supernatants were carefully discarded. Pellets were left to air dry and resuspended in 500 µl – 1 ml dH_2O.

2.1.10 Site directed mutagenesis

Amino acid changes were carried out using the Quikchange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers instructions.

2.1.11 DNA constructs

Expression vector pEGFP was obtained from Clontech. Table 2.1 lists constructs that were used throughout this research that were kindly provided by other members of the lab or from other laboratories.

Construct	Vector	Insert	Source of Gift
Myc-AIP4 (Itch)	pRK5	Itch, full-length, human	Kindly donated by Dr. D. Birnbaum (Courbard et al., 2002)
FLAG-δ-catenin	pCMV5	δ-catenin, full-length, human	Kindly donated by Dr. S. Nakamura (Fujita et al., 2004)
Myc-Rho T17N	pRK5	RhoA, full-length, Dominant Active, human	Kindly donated by Prof. A. Hall
Myc-Cdc42 Q61L	pRK5	Cdc42, full-length, Dominant Active, human	Kindly donated by Prof. A. Hall
Myc-Rac G12V	pBOS	Rac, full-length, Dominant Active, human	Kindly donated by Prof. A. Hall
AU-Mena	pBABE	Enabled, full-length, mouse	Kindly donated by Prof. A. Hall
HA-E-cadherin	pcDNA3.1	E-cadherin, full-length, mouse	Kindly donated by Prof. W. Birchmeier
v-Src	pSG	Src, full-length, mouse	Kindly donated by Prof. W. Birchmeier
FLAG-Glis2	pcDNA3.1	Glis2, full-length, mouse	Prepared by N. Serpente, Fujita lab
HA-Glis2	pCAGGS	Glis2, full-length, mouse	Prepared by N. Serpente, Fujita lab
FLAG-p120	pcDNA3.1	p120 isoform 1B, full-length, mouse	Prepared by N. Serpente, Fujita lab
HA-p120	pcDNA3.1	p120 isoform 1B, full-length, mouse	Prepared by N. Serpente, Fujita lab
HA-p120 (N-term)	pcDNA3.1	p120 isoform 1B, amino acids 1-647	Prepared by N. Serpente, Fujita lab
HA-p120 (C term)	pcDNA3.1	p120 isoform 1B, amino acids 385-911	Prepared by N. Serpente, Fujita lab
FLAG-Hakai	pcDNA3.1	Hakai, full-length, mouse	Prepared by N. Serpente, Fujita lab
HA-Hakai	pcDNA3.1	Hakai, full-length, mouse	Prepared by N. Serpente, Fujita lab
FLAG-RFP	pcDNA3.1	Ret Finger Protein, full-length, mouse	Prepared by N. Serpente, Fujita lab
HA-Ub	pcDNA3.1	Ubiquitin, full-length, mouse	Prepared by N. Serpente, Fujita lab
FLAG-GLI1	pcDNA3.1	GLI1, full-length, human	Prepared by N. Serpente, Fujita lab

Table 2.1 *Details of constructs used during this research that were obtained from other laboratories or prepared by other members of the Fujita Lab.*

Table 2.2 describes a series of constructs prepared by PCR and subcloning in which the template for PCR was pcDNA FLAG-Glis2.

Construct	Insert	Primers
Glis2 ΔC1	Glis2, aa 1-257	**Primer 1:** 5'GGAATTCGCGGCCGCGCATGCACTCCTTGGACGAGCCCC3' **Primer 2:** 5'GGAATTCGCGGCCGCTTAGTGGGAGCGGTTGTGGTAG3'
Glis2 ΔC2	Glis2, aa 1-290	**Primer 1: same as above** **Primer 2:** 5'GGAATTCGCGGCCGCGTGGGTACGGGTGTGCTTG3'
Glis2 ΔC3	Glis2, aa 1-307	**Primer 2:** 5'GGAATTCGCGGCCGCGGGGTCCGTGTAACGCTTGTG3'
Glis2 ΔC4	Glis2, aa 1-317	**Primer 2:** 5'GGAATTCGCGGCCGCTTAATGGGCCTTGATGTGTTTGCGC3'
Glis2 ΔC5	Glis2, aa 1-324	**Primer 2:** 5'GGAATTCGCGGCCGCGGGCGCAGCTGCAGGAGCTCC3'
Glis2 ΔC6	Glis2, aa 1-357	**Primer 2:** 5'GGAATTCGCGGCCGCTTAGTTCGGGATGATGATCTGAGCCCC3'
FLAG-Glis2-Myc	N-terminally FLAG tagged, C-terminally Myc tagged Glis2, full-length	**Primer 2:** 5'CGGGCCCAAGCTTGTTTACCACAGCTGGTTTGAGCAGC3'

Table 2.2 *Constructs in which the template for PCR was FLAG-Glis2.*

Table 2.3 describes constructs that were prepared by site-directed mutagenesis that used pcDNA-FLAG-Glis2 as a template.

Table 2.4 describes a series of constructs of putative Hakai-binding proteins prepared by subcloning from the yeast expression vector pACTII into mammalian expression vectors pcDNA and pGEX, and Table 2.5 describes constructs prepared by amplifying the open reading frame (ORF) of clones from the pACTII vector and subcloning into pcDNA-FLAG.

Construct	Insert	Primers
Glis2 Y291A	pcDNA FLAG Glis2, full-length **Mutation:** aa 291, Tyrosine/Alanine	**Primer 1:** 5'GCACACCCGTACCCACGCCGTAGACAAGCCC3' **Primer 2:** 5'GGGCTTGTCTACGGCGTGGGTACGGGTGTGC3'
Glis2 D293A	pcDNA FLAG Glis2, full-length **Mutation:** aa 293, Glutamate/Alanine	**Primer 1:** 5'CCCGTACCCACTACGTAGCCAAGCCCTACTACTGC3' **Primer 2:** 5'GCAGTAGTAGGGCTTGGCTACGTAGTGGGTACGGG3'
Glis2 DAKA	pcDNA FLAG Glis2, full-length **Mutation:** aa 293-4, DK/AA	**Primer 1:** 5'CCCGTACCCACTACGTAGCCGCGCCCTACTACTGC3' **Primer 2:** 5'GCAGTAGTAGGGCGCGGCTACGTAGTGGGTACGGG3'
Glis2 ZnF	pcDNA FLAG Glis2, full-length **Mutation:** aa 170, Cysteine/Alanine	**Primer 1:** 5'CCAAGCAGCTGGTGGCTCGATGGGCCAAG3' **Primer 2:** 5'CTTGGCCCATCGAGCCACCAGCTGCTTGG3'

Table 2.3 *Details of constructs that were prepared by site-directed mutagenesis.*

Construct	Insert	Restriction Enzymes
pGEX-HH5	Clone HH5, fragment coding for SH3 domain of IRSp53	XhoI
pcDNA-FLAG-HH31	Clone HH31, coding for Itch	NotI, XhoI
pGEX-HH31	Clone HH31, coding for Itch	XbaI, BGLII, T4; ligated into pGEX (SmaI, CIP)
pcDNA-FLAG-HH70	Clone HH70, coding for NADH DH4	NotI, XhoI
pcDNA-FLAG-HH82	Clone HH82, coding for AES	NotI, XhoI
pcDNA-FLAG-HH97	Clone HH97, coding for Mena	NotI, XhoI
pGEX-HH97	Clone HH97, coding for Mena	EcoRI, XhoI
pcDNA-FLAG-HH99	Clone HH99, coding for GZP1	NotI, XhoI
pcDNA-FLAG-HH101	Clone HH101, coding for KSRP	EcoRI, XhoI

Table 2.4 *Constructs of Hakai-binding partners prepared by restriction digests of yeast 2-hybrid clones in the pACTII vector. All fragments were cloned into indicated vectors that had been cut with the same enzymes, followed by CIP treatment, except pGEX-HH31, as indicated.*

Construct	Insert	Primers
pcDNA-FLAG-HH5	ORF of HH5, coding for IRSp53	**Primer 1:** 5'GGAATTCGCGGCCGCACATGTCTTTGTCTCGCTCAGAGGAG3' **Primer 2:** 5'GGAATTCGCGGCCGCTCACACTGTGGACACCAGCGTGCC3'
pcDNA-FLAG-HH9	ORF of HH9, coding for E1B-AP5	**Primer 1:** 5'GGAATTCGCGGCCGCCCATGAGTGAACAGAGTATCTGTCAGGC3' **Primer 2:** 5'GGAATTCGCGGCCGCTTATGCAGTGTTCGACTTGCTCAGCTCC3'

Table 2.5 *Constructs of Hakai-binding partners prepared by PCR amplification of ORFs from yeast 2-hybrid clones in the pACTII vector.*

Table 2.6 describes constructs prepared by annealing siRNA oligonucleotides (oligos) into the pSuperGFP vector. Short hairpin oligos targeting all isoforms of monkey p120 were designed using an online design tool (oligoengine.com). Table 2.7 describes siRNA oligos used in transient transfections. These siRNA constructs for transient expression were designed using an online tool (qiagen.com).

Name of Construct	Sequence
pSuperGFP-p120 Oligo 1	**Sense:** 5'GATCCCCGACCTCAGATGATGGGACCTTCAAGAGAGGTCCCATCATCTGAGGTCTTTTTA3' **Antisense:** 5'AGCTTAAAAAGACCTCAGATGATGGGACCTCTCTTGAAGGTCCCATCATCTGAGGTCGGG3'
pSuperGFP-p120 Oligo 2	**Sense:** 5'GATCCCCCAGGAGCTATGAAGACATGTTCAAGAGACATGTCTTCATAGCTCCTGTTTTTA3' **Antisense:** 5'AGCTTAAAAACAGGAGCTATGAAGACATGTCTCTTGAACATGTCTTCATAGCTCCTGGGG3'

Table 2.6 *Constructs prepared using pSuperGFP.*

Name of Oligo	Sequence	Target species
p120 1	**Sense:** r(CCGCUCAGAAAAAGAAGUU)dTdT **Antisense:** r(AACUUCUUUUUCUGAGCGG)dTdT	Monkey/Human
p120 2	**Sense:** r(CUGCGGAAGCCACUGGAAA)dTdT **Antisense:** r(UUUCCAGUGGCUUCCGCAG)dTdT	Monkey/Human
Glis2 1	**Sense:** r(GGUACAAGAUGCUCAUCCA)dTdT **Antisense:** r(UGGAUGAGCAUCUUGUACC)dTdT	Human
Glis2 2	**Sense:** r(CGGAAACUCUUCUGUGAAA)dTdT **Antisense:** r(UUUCACAGAAGAGUUUCCG)dTdT	Human

Table 2.7 *siRNA oligos from Qiagen used for transient transfections.*

2.1.12 DNA quantitation

The concentration and purity of DNA preparations were examined by optical density ($Abs_{260/280}$) and gel electrophoresis.

2.1.13 DNA sequencing

All constructs cloned were verified by automated DNA sequencing (MWG Biotech).

2.2 CELL BIOLOGY

All tissue culture plasticware was purchased from Nunc.

2.2.1 Reagents

Table 2.8 describes the primary antibodies that were used during this research. Details of the stock solutions and working concentrations for immunofluorescence and western blotting are provided.

Primary Antibodies	Stock Conc.	Working Conc. (Western blotting)	Working Conc. (Immunofluorescence)
Mouse anti-FLAG, Clone M2 (Sigma)	4.9 mg/ml	-	1:100
Mouse anti-FLAG, Clone M2 POD (Sigma)	1 mg/ml	1:5000	-
Mouse anti-Myc clone 4A6 (Upstate)	0.3 mg/ml	1:2000	-
Rat anti-HA Clone 3F10 (Roche)	0.2 mg/ml	1:2000	1:100
Anti-HA-POD Clone 3F10 (Roche)	25 µg	1:2000	-
Mouse anti-pp120 (BD Biosciences)	0.25 mg/ml	1:1000	1:100
Mouse anti-E-cadherin (BD Transduction Laboratories)	0.25 mg/ml	1:1000	-
Rabbit anti-Glis2 (Eurogentec)	Antiserum	1:1000	1:100
Rabbit anti-GFP (Molecular Probes)	Unknown	-	1:1000
Mouse anti-Lim 1/2 Clone 4F2 (Developmental Studies Hybridoma Bank, DSHB)	Unknown	-	1:100
Mouse anti-BrdU (DSHB)	Unknown	-	1:200
Mouse anti-Nkx 2.2 Clone 74.5A5 (DSHB)	Unknown	-	1:20
Mouse anti-Pax 6 (DSHB)	Unknown	-	1:100
Mouse anti-GAPDH (Chemicon)	1 mg/ml	1:200	-
Mouse anti-MAPK1 (Qiagen)	100 µg/ml	1:1000	-
Rat anti-tubulin (ImmunologicalsDirect)	Undisclosed	1:200	-
Anti-CBP (Santa Cruz)	200 µg/ml	1:1000	-

Table 2.8 *Details of primary antibodies used in Western blotting and immunofluorescence experiments.*

Table 2.9 describes the inhibitors that were used during this research. Details of the stock solutions and treatment conditions are provided. In addition, epidermal growth factor (EGF) was used to treat cells at 50-100 ng/ml for 4 h.

Inhibitor	Function	Treatment
Nocodazole (Calbiochem)	Inhibits microtubule polymerisation by sequestering tubulin	5 μg (10 μl of 5 mg/ml stock) added to 10 ml DMEM culture medium, and incubated for 4 hr.
MG132 (Sigma)	Proteasome (20S and 26S subunits) inhibitor.	4 μl of 50 mM stock (20 μM final conc) added to 10 ml DMEM culture medium and incubated for 6 hr.
Lactacystin (Calbiochem)	Proteasome (20S subunit) inhibitor.	10 μl of 5 mM stock (5 μM final conc) added to 10 ml DMEM culture medium and incubated for 6 hr.
Orthovanadate (5 mM sodium vanadate and 5 mM H_2O_2 incubated for 20 min at RT)	Inhibitor of tyrosine phosphatases. Induces hyperphosphorylation of proteins.	1 ml of stock added to 10 ml DMEM culture medium and incubated for 1-2 hr.

Table 2.9 *Details of inhibitors used during this research, including their functions and the conditions for treatments.*

2.2.2 Cell lines and culture conditions

The immortalised, transformed cell lines Human Embryonic Kidney (HEK) 293, monkey fibroblast COS-1, murine L fibroblast cells (from the Birchmeier laboratory, Berlin) and human mammary adenocarcinoma MDA-MB-231 (kindly donated by A. Jaffe, Hall laboratory) were cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco) supplemented with 10% complete fetal calf serum (FCS, PAA laboratories), 1% penicillin/streptomycin (Gibco) and 1% Glutamax^{TM} (Gibco). Cells were grown in incubators at 37°C in 5% CO_2. All solutions for use on live cells were pre-warmed to 37°C prior to use. Lysates of MDCK (canine kidney epithelial) and MCF-7 (human breast carcinoma) cells were kindly provided by members of the lab.

2.2.3 Freezing and thawing cultured cells

Aliquots of $1x10^7$ cells were suspended in 2 ml of DMEM supplemented with 20% FCS and pen/strep. These were frozen overnight at -20°C. The aliquots were moved to -80°C for a further 8 hrs before being stored permanently under liquid nitrogen. Individual aliquots were thawed rapidly at 37°C and placed in a 15 ml tube with 10 ml pre-warmed medium. After centrifugation at 200 x g for 5 min, cells were plated in 90 mm culture dishes and were incubated overnight to allow adhesion to occur. Cells were then grown in fresh medium, and initially passaged two days post-thawing.

2.2.4 Maintenance of HEK293, COS-1 and MDA-MB-231 cells

Cultures were passaged when they reached 80-90% confluency. Cells were washed twice in sterile phosphate-buffered saline (PBS) and removed from dishes using 1 ml

trypsin/EDTA (Gibco) per 90 mm culture dish for 2 min at 37°C. Flasks were gently tapped and the cells were resuspended in 9 ml of culture medium. New dishes were prepared by addition of 9 ml medium to 1 ml cell suspension.

2.2.5 Transfection of HEK293 cells using Ca_2PO_4

Cells were seeded in 90 mm dishes ($1x10^6$ cells per dish). 24 hr after seeding, cells were checked to ensure confluency was between 30-50%. Plasmid DNA (maximum of 15 μg per reaction) was added to 450 μl dH_2O and 50 μl 2.5 M $CaCl_2$ and mixed thoroughly. The DNA mixture was added dropwise to 500 μl of 2x Hepes buffer (42 mM HEPES, 2 mM $Na_2HPO_4 \bullet 2H_2O$, 0.5 M NaCl, 20 mM KCl, pH 7.05, 0.22 μm filtered), while vortexing. The DNA/Ca_2PO_4 preparation was left to stand for 5 min and then added dropwise to the cells with vortexing. Cells were incubated for 4-6 hours before the medium was changed. 24 hrs after transfection, cells were used for immunoprecipitation and immunofluorescence experiments.

2.2.6 Transfection of COS-1 using Lipofectamine 2000™ reagent

Cells were seeded in 90 mm dishes ($2x10^6$ cells per dish) so that they were 50-90% confluent at time of transfection. Cells were transfected with Lipofectamine 2000™ reagent (Invitrogen) according to the manufacturers instructions.

2.2.7 Transfection of HEK293 and COS-1 cells using Hi-PerFect® reagent

siRNA samples were prepared by adding 250 μl siRNA Suspension Buffer (Qiagen) to each tube containing 5 nmol lyophilised siRNA, to give a 20 μM solution. Tubes were then heated for 1 min at 90°C, and then incubated at 37°C for 1 h. Samples were stored at -20°C. Cells were seeded in 24 well plates ($5x10^4$ cells per well) 24 hrs prior to transfection. Immediately before transfection, cells were placed in fresh serum free medium supplemented with pen/strep. 1.5 μg (6 μl) of each siRNA oligo and 9 μl of Hi-PerFect® reagent (Qiagen) was added to 100 μl serum free, antibiotic free DMEM. RNAi mixtures were incubated at RT for 15 min, and each suspension was then added dropwise to a well of the culture plate. The cells were incubated at 37°C, the medium was replaced with normal culture medium after 24 hrs, and cells were then incubated for a further 72 hrs. Table 2.7 describes siRNA oligos that were used in these experiments.

2.2.8 Immunofluorescence microscopy

Cells were seeded onto acid-washed glass coverslips (13 mm, No. 1½, BDH) and transfected 24 hours prior to preparation for immunofluorescence. Cells were washed 3x in PBS, fixed in 1 ml 3% paraformaldehyde-PBS for 15 min. After washing twice in PBS, cells were permeabilised in 0.5% Triton X-100-PBS for 15 min, followed by blocking in DMEM containing 10% FCS for 1 hr. They were further incubated in primary antibody for 1 hr, washed 4x in PBS, incubated in Cytochrome 2 (Cy2) or Cy3-conjugated secondary antibody (Jackson Immunochemicals) solution for 1 hr, and washed 4x in PBS. Coverslips were then immersed in Hoechst (1:5000 dilution, Molecular Probes) for approximately 1 min and washed 2x in PBS. The coverslips were then mounted onto glass slides using Mowiol (5 µl/coverslip, Calbiochem). Cells were examined using a Nikon optiphot-2 microscope and a Nikon 40x 1.0 oil-immersion objective with a Bio-Rad MRC 1024 laser and Bio-Rad LaserSharp confocal software system.

2.3 PROTEIN BIOCHEMISTRY

2.3.1 SDS polyacrylamide gel electrophoresis

Polyacrylamide gels were prepared according to details in Table 2.10, using reagents from Sigma. N,N,N,N-tetramethyl-ethylenediamine (TEMED, National Diagnostics) and ammonium persulphate (APS, Bio-Rad) were used to induce polymerisation.

	10%	10% (mini)	Stacking	Stacking (mini)
dH_2O	12 ml	3 ml	9.5 ml	3.2 ml
1.5 M Tris/Cl pH 8.8	10 ml	2.5 ml	-	-
0.5 M Tris/Cl pH 6.8	-	-	3.75 ml	1.25 ml
50% Glycerol	8 ml	2 ml	-	-
40% Acrylamide	10 ml	2.5 ml	1.5 ml	500 µl
10% SDS	400 µl	100 µl	150 µl	50 µl
TEMED	40 µl	10 µl	15 µl	5 µl
10% APS	100 µl	25 µl	150 µl	50 µl

Table 2.10 *Constituents of polyacrylamide running and stacking gels.*

Samples were mixed with protein sample buffer and heated to 95°C for 10 min prior to loading. Gels were run in Bio-Rad Protean II™ or Mini Protean II™ tanks using SDS-

PAGE running buffer (National Diagnostics). Gels were run at 9 mA (one gel) or 18 mA (two gels) overnight at RT. Full range rainbow markers (Amersham) were loaded as molecular weight markers.

2.3.2 Coomassie staining of polyacrylamide gels

Polyacrylamide gels were stained using gentle agitation for a minimum of 1 hr in acrylamide gel stain (0.04% w/v Brilliant blue R, 5% v/v methanol and 5% v/v glacial acetic acid). Gels were destained using several changes of polyacrylamide gel destain (40% methanol and 10% glacial acetic acid) until the proteins were clearly visible and the background was clear. Gels were dried onto Whatmann 3M filter paper using a heated, vacuum-assisted gel drier (Bio-Rad).

2.3.3 Western blotting

Western blotting was carried out using a Bio-Rad Trans-Blot® electrophoretic transfer cell. Polyvinylidene difluoride (PVDF, Millipore) membranes were soaked in methanol and then shaken in cold transfer buffer (25 mM Tris, 192 mM glycine and 10% methanol) until all drops of methanol had dispersed. Sponges and Whatmann 3M filter paper were also soaked in transfer buffer prior to use. A pocket from the transfer apparatus was placed clear side down on a tray. A soaked sponge was added first, followed by 3 sheets of filter paper. These were pressed down with a pipette to remove air bubbles. The PVDF membrane was then added, followed by the gel, 3 more sheets of filter paper, pressing down after each sheet, and another sponge. The pocket was closed, placed in the transfer apparatus and covered in transfer buffer. Transfer was carried out either overnight at 220 mA at 4°C, or for 2 hrs at 70 V at 4°C.

Membranes were blocked by shaking in 3% 'Marvel' milk powder-PBS for 1 hr at RT. Primary antibody or peroxidase-conjugated FLAG- or HA-tagged antibodies were added (see Table 2.8) for either 1 hr at RT or overnight at 4°C with agitation. Membranes incubated with peroxidase-conjugated primary antibodies were washed 3 times for 10 min at RT in PBS-Tween® (0.05% v/v Tween® 20, Sigma), and bands were visualised using ECL western blotting detection reagents (Amersham) and high performance chemiluminescence film (Hyperfilm, Kodak) according to the manufacturers instructions. Membranes incubated with unconjugated primary antibody were washed 5 times for 10 min at RT in PBS-Tween® and reblocked in 3% milk-PBS

for 30 min followed by addition of peroxidase conjugated secondary antibodies (Jackson Immunochemicals) for 1 hr at RT with agitation. Secondary antibodies were washed off using 3x 10 min washes in PBS-Tween®. Bands were then visualised as described above.

2.3.4 Preparation of GST fusion proteins

BL21 *E.coli* were transformed with the required pGEX construct and spread onto agar plates containing 50 μg/ml ampicillin. Plates were inverted and incubated at 37°C overnight. A single colony was inoculated into 3 ml of LB containing 50 μg/ml ampicillin, and several hours later the 3 ml culture was further diluted into 200 ml, which was grown overnight at 37°C with agitation. 20 ml of the overnight culture was used to dilute 750 ml of LB plus 50 μg/ml ampicillin in four flasks (a total of 3 L). These cultures were grown at 37°C with agitation until the OD_{600} reading was between 0.3-0.4. Fusion protein production was induced by addition of 750 μl of isopropyl-B-D-thiogalactopyranoside (IPTG, Melford Laboratories Ltd) to each flask. Cultures were incubated for 4 hours at 30°C with gentle agitation and the cells were then harvested at 3400 x g for 20 min. The cells were washed once with 40 ml PBS and pellets were stored overnight at -80°C.

The cells were thawed on ice, resuspended in 20 ml lysis buffer (20 mM Tris/Cl pH7.5, 10% (w/v) Sucrose (Sigma), 1 mM Dithiothreitol (DTT, Sigma), 1 mM phenyl methyl sulphonyl fluoride (PMSF, Sigma), made up with dH_2O), and sonicated on ice (amplitude 5 microns) in five 30 sec bursts, with a 1 min interval between each pulse, using a Soniprep 150 (Sanyo). The lysate was centrifuged at 50,000 x g for 1 hr at 4°C. The supernatant was carefully removed and filtered through a 0.45 μm filter and then transferred to 50 ml falcon tube containing 1 ml of a 50% glutathione-Sepharose™ 4B bead (Amersham) slurry. The suspension was rotated for 1 hr at 4°C, followed by affinity chromatography. After application of the suspension, the affinity column was washed twice with wash buffer (20 mM Tris/Cl pH 7.5, 1 mM DTT) and eluted with pH 8 elution buffer (20 mM Tris/Cl pH 7.5, 50 μmol Glutathione (GSH, Sigma), made immediately prior to use). Aliquots were stored as 200 μl aliquots at -80°C. Protein concentration was determined by SDS-PAGE with BSA standards.

2.3.5 GST pull-downs

HEK293 cells were transfected 24 hrs prior to immunoprecipitation as described previously (Section 2.2.5). Cells were scraped in 1 ml ice-cold PBS, and centrifuged at 400 x g for 3 min at 4°C. Cells were lysed in 1 ml cold lysis buffer (20 mM Tris/Cl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100) supplemented with 5 μg ml^{-1} leupeptin (Sigma), 50 mM PMSF and 7.2 trypsin inhibitor units ml^{-1} of aprotinin (Sigma), and then vortexed briefly. Cells were rotated for 30 min at 4°C. After clarification of the lysates by centrifugation at 5000 x g, aliquots were taken to assess total protein levels prior to GST pulldowns. The remaining supernatants were added to microfuge tubes containing 100 μl of a 50% slurry of glutathione-Sepharose™ 4B conjugated to GST alone or the GST-fusion protein (prepared by coupling 30 μl of glutathione-Sepharose™ beads with 20 μg of GST/GST-fusion protein per reaction in a total volume of 500 μl of PBS at 4°C with rotation; beads were washed twice with lysis buffer). Supernatants were rotated with glutathione beads for 1 hr at 4°C, and then collected by centrifugation at 5000 x g for 1 min at 4°C. The beads were washed twice in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were boiled for 10 min at 95°C then centrifuged for 10 sec at 5000 x g. The samples were analysed by SDS-PAGE, Coomassie staining and Western blotting.

2.3.6 Immunoprecipitations

HEK293 and COS-1 cells were transfected 24 hrs prior to immunoprecipitation with Ca_2PO_4 treatment or Lipofectamine 2000™ respectively. Cells were scraped in 1 ml ice-cold PBS, and centrifuged at 400 x g for 3 min at 4°C. Cells were lysed in 1 ml cold lysis buffer (20 mM Tris/Cl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100) supplemented with 5 μg ml^{-1} leupeptin, 50 mM PMSF and 7.2 trypsin inhibitor units ml^{-1} of aprotinin, and then vortexed briefly. Cells were rotated for 30 min at 4°C. After clarification of the lysates by centrifugation at 5000 x g, aliquots were taken to assess total protein levels prior to immunoprecipitations. The remaining supernatants were added to microfuge tubes containing 100 μl of a 50% slurry of protein G-Sepharose™ 4B (Amersham) conjugated to the immunoprecipitating antibody (prepared by coupling 30 μl of Protein G beads with 5 μl of antibody per reaction in a total volume of 500 μl of PBS at 4°C with rotation; beads were washed twice with lysis buffer). Supernatants were rotated with antibody beads for 1 hr at 4°C, and then collected by

centrifugation at 5000 x g for 1 min at 4°C. The beads were washed twice in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were boiled for 10 min at 95°C then centrifuged for 10 sec at 5000 x g. The samples were analysed by SDS-PAGE and Western blotting.

For immunoprecipitations using 0.25% SDS lysis buffer, cells were treated as follows. HEK293 cells were transfected 24 hrs prior to immunoprecipitation with Ca_2PO_4 treatment. Cells were scraped in 1 ml ice-cold PBS, and centrifuged at 400 x g for 3 min at 4°C. Cells were lysed in 0.5 ml cold SDS lysis buffer (0.25% (w/v) SDS, 20 mM Tris/Cl pH 7.5) supplemented with 1.25 mg/ml N-ethylmaleimide (NEM, Sigma), 5 μg/ml leupeptin, 50 mM PMSF and 7.2 trypsin inhibitor units/ml of aprotinin, and then sonicated for 20 sec at an amplitude of 5 microns. Cells were rotated for 30 min at 4°C. After clarification of the lysates by ultracentrifugation at approximately 450,000 x g (100,000 rpm using a Beckman TLA 100.3 rotor), aliquots were taken to assess total protein levels prior to immunoprecipitations. The remaining supernatants were diluted to 0.1% SDS and added to microfuge tubes containing 100 μl of a 50% slurry of protein G-Sepharose™ 4B (Amersham) conjugated to the immunoprecipitating antibody (prepared by coupling 30 μl of Protein G beads with 5 μl of antibody per reaction in a total volume of 500 μl of PBS at 4°C with rotation; beads were washed twice with lysis buffer). Supernatants were rotated with antibody beads for 1 hr at 4°C, and then collected by centrifugation at 5000 x g for 1 min at 4°C. The beads were washed twice in 0.1% SDS lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were boiled for 10 min at 95°C then centrifuged for 10 sec at 5000 x g. The samples were analysed by SDS-PAGE and Western blotting.

2.3.7 Nuclear fractionations

Cells were washed twice with PBS and trypsinised thoroughly until well separated. The cells were then scraped in PBS and centrifuged at 400 x g for 3 min. Pelleted cells were resuspended in 150 μl 2x lysis buffer (50 mM Hepes/NaOH pH 7.4, 10 mM EGTA, 5 mM $MgCl_2$, 20% Glycerol, 2% NP-40 and 2 mM DTT) containing 5 μg ml^{-1} leupeptin, 50 mM PMSF and 7.2 trypsin inhibitor units ml^{-1} of aprotinin. Cells were then immediately triturated with a 25G needle 12 times. After centrifugation at 110 x g for 5 min at 4°C, the supernatant was removed as the cytoplasmic fraction, and

remaining nuclei were washed twice in 1x lysis buffer. The supernatant and nuclear fractions were then boiled for 10 min with SDS-PAGE sample buffer and examined by Western blotting. Purity of nuclear fractions was confirmed by immunoblotting with anti-CBP (CREB-binding protein), anti-N-cadherin and anti-tubulin antibodies (Figure 2.1).

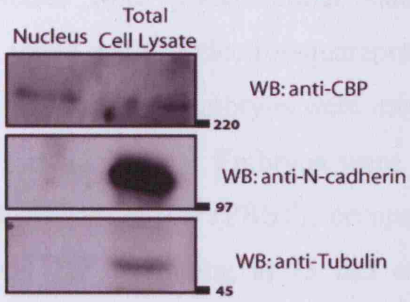

Figure 2.1 Confirmation of purity of nuclear fractions. *Lysates of HEK293 cells were nuclear fractionated and samples were examined by Western blotting using anti-CBP antibody as a nuclear marker, anti-N-cadherin as a membrane marker, and anti-tubulin as a cytoplasmic marker. By kind permission of E. Ferber.*

2.3.8 DNA-Cellulose binding assays

For DNA-cellulose binding assays, cells were lysed in a buffer containing 10 mM Hepes/NaOH pH 7.4, 0.1% Triton X-100, 100 mM NaCl, 10% glycerol, and 0.05 mM EDTA supplemented with 5 μg ml^{-1} leupeptin, 50 mM PMSF and 7.2 trypsin inhibitor units ml^{-1} of aprotinin, and clarified by centrifugation at 5000 x g for 20 min. 50 μl of cell lysate (approx 100 μg protein) was incubated with 25 μl DNA-cellulose (GE Healthcare) or cellulose (Sigmacell cellulose type 50; Sigma) in 100 μl of cold lysis buffer for 1 h at 4°C. For competition assays, lysates were pre-incubated with the stated amounts of DNA (sonicated, calf thymus; GE Healthcare) or RNA (calf-liver type IV; Sigma) and rotated for 30 min at 4°C, and then added to 25 μl of DNA-cellulose beads. Following incubation, the beads were washed four times with 1 ml lysis buffer, and the amount of protein retained on the beads were determined by Western blotting.

2.3.9 Protein concentration quantification

To ensure equal loading on polyacrylamide gels, the protein concentration of lysates was quantitated using the D_C Protein Assay reagent (Bio-Rad) and measured on a VERSAmax microplate reader (Molecular Devices).

2.4 CHICK ELECTROPORATION

pcAGGS-HA-p120, pcAGGS-FLAG-Glis2 and pcAGGS-FLAG-Glis2-ΔC were expressed in chick embryos with pCAGGS-IRES-GFP by electroporation. The indicated plasmids were injected into Hamburger and Hamilton (1951) (HH) stage 10-12 chick embryos. Electrodes were placed either side of the neural tube and electroporation carried out using a T820 electro-squareporator (BTX Inc.) delivering five 50 msec pulses of 30 V. Transfected embryos were incubated at 38°C for 48 h and fixed and processed at HH stage 20-24. Embryos were fixed for 1 h in 4% PFA, washed in PBS with 0.1% Triton X-100 (PBST), cryoprotected in 30% sucrose in PBS, and imbedded in OCT for sectioning at 15 μm on a cryostat. For antibody stainings, slides were air-dried, washed twice with PBST, incubated with PBST plus 1% bovine serum albumin (BSA) for 10 min, and incubated with the primary antibody in PBST with 1% BSA overnight at 4°C. Slides were then washed three times with PBST and incubated with FITC- or Cy2-conjugated secondary antibodies in PBST with 1% BSA. Slides were then dehydrated and mounted with DAPI. Samples were imaged using a Zeiss LSM510 confocal microscope.

2.5 COMPUTATIONAL SOFTWARE

All image analysis was performed using Adobe Photoshop, version 6.0. Quantitative data was depicted using Microsoft Excel and Quantity One software. Sequence alignments were performed using Clustal W software. Student *t* tests assuming paired variances were performed using Microsoft Excel.

CHAPTER 3

CHARACTERIZATION OF

GLIS2 BINDING TO p120

3.1 INTRODUCTION

To better understand signalling downstream of cadherins, an analysis of the cadherin-binding protein p120 was undertaken. As described in Section 1.3, p120 has been shown to be involved in a number of important processes within cells. In epithelial cells, p120 is predominantly localized at cadherin-based cell-cell contact sites, and is also associated with microtubules (Chen et al., 2003; Franz and Ridley, 2004; Yanagisawa et al., 2004). However, under several experimental conditions, its nuclear localization has also been observed, suggesting a functional role for p120 in the nucleus (Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). Indeed, p120 interacts with Kaiso, a BTB/POZ domain zinc finger transcriptional repressor (Daniel and Reynolds, 1999; Prokhortchouk et al., 2001). This precedent of a nuclear role for p120 encourages further investigation of the involvement of p120 in signalling to the nucleus.

This chapter describes the identification and characterization of a novel binding partner of p120, Glis2. Glis2 is a transcriptional repressor that is related to the GLI/Ci family of transcription factors involved in Sonic Hedgehog (Shh) signalling (Section 1.4, Introduction). The interaction of p120 with another transcription factor (in addition to Kaiso) points to further roles for p120 in the nucleus. Here, the cleavage of the Glis2 protein in the presence of p120 is described and investigated under different experimental conditions.

3.2 IDENTIFICATION OF GLIS2 AS A p120-INTERACTING PROTEIN

3.2.1 Glis2 interacts with p120 in a yeast 2-hybrid assay

A yeast 2-hybrid assay was performed by Dr. Y. Fujita (2001) using full-length p120 as bait to identify new potential components of cadherin-based signalling pathways. Briefly, DNA encoding the 911 amino-acid mouse p120 protein isoform 1B (see Figure 1.4, Introduction) was subcloned into a 'bait' yeast expression vector containing the LexA DNA binding domain, and was used to screen a Hollenberg (mouse embryo) cDNA library, which had been subcloned into a 'prey' expression

vector containing the VP16 activation domain (proof of concept described in (Fields and Song, 1989).

LexA is a transcription factor which regulates the transcription of genes involved in the SOS response in *E. coli* (Little and Mount, 1982), but can be manipulated to activate transcription from the histidine (HIS3) and β-galactosidase (lacZ) promoters in the L40 yeast strain. This is achieved by expression in this strain of the lacZ and HIS3 coding sequences driven by minimal GAL1 promoters fused to multimerized LexA binding sites (Two Hybrid Library Screen protocol, Dr. Stan Hollenberg, 1993). The DNA-binding domain of LexA can bind to cognate promoters, but cannot activate transcription. VP16 is also a bacterial transcription factor, and a fragment is used in yeast two-hybrid screens that can strongly activate transcription, but lacks the DNA binding domain (Two Hybrid Library Screen protocol, Dr. Stan Hollenberg, 1993). An interaction between bait and prey proteins fused to the LexA DNA binding domain and VP16 activation domain, respectively, results in transcriptional activation of histidine and β-galactosidase, leading to growth of colonies on selective media (Figure 3.1A; (Vojtek et al., 1993). In this way, the transcription factor Glis2 was identified as a novel binding partner for p120 (Figure 3.1B).

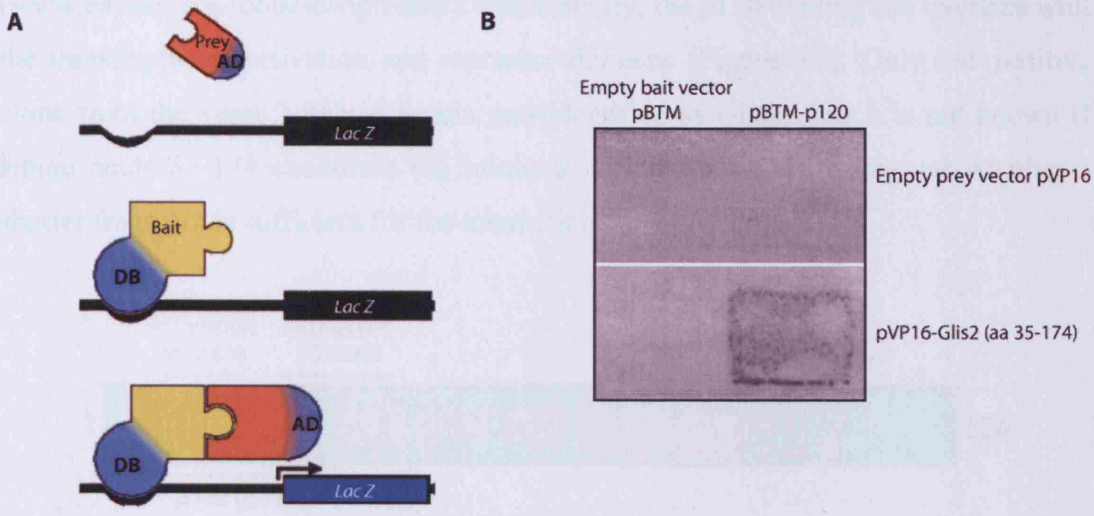

Figure 3.1 Yeast 2-hybrid assay. A *Principle of the 2-hybrid system. Two chimeras, one containing the DNA-binding domain (DB: blue circle) and one that contains an activation domain (AD: blue semi-circle), are co-transfected into an appropriate host strain. If the fusion partners (yellow and red) interact, the DB and AD are brought into proximity and can activate transcription of reporter genes (here LacZ). Diagram from (Van Criekinge and Beyaert, 1999)* ***B*** *Glis2 interacts with p120 by yeast 2-hybrid. The* S. cerevisiae *reporter strain L40 was transformed with empty bait vector pBTM or pBTM-p120 in conjunction with either empty prey vector pVP16 or the Hollenberg cDNA library isolate pVP16-Glis2 (amino acids 35-174) on selective media lacking the amino acid histidine. The plate was incubated at 30°C for 3 days. By kind permission of Y. Fujita.*

3.2.2 Structure of Glis2

Glis2 is a Krüppel-like protein (see Section 1.4, Introduction) that has a tandem of five Cys_2-His_2-type zinc finger domains in the central region (domain structure shown in Figure 3.2; (Lamar et al., 2001; Zhang and Jetten, 2001). Zinc finger motifs are characteristic of proteins that bind DNA, and the precise structure of the zinc finger confers specificity to the protein-DNA interaction (Klug and Schwabe, 1995). The p120-binding site of Glis2 (amino acids 35-174), determined by yeast 2-hybrid analysis, is located N-terminally to the zinc finger domain. Previous studies have reported the existence of an activation domain from amino acids 71-137, and a repressor domain from amino acids 151-194 that overlaps with the first zinc finger (Figure 3.2). The N and C termini of Glis2 did not exhibit any further classic domain

motifs when the sequence was analysed using an online structure prediction tool (www.expasy.org/tools/scanprosite/). Interestingly, the p120 binding site overlaps with the transcriptional activation and repressor domains (Figure 3.2). Only one positive clone from the yeast 2-hybrid screen was identified as Glis2, thus it is not known if amino acids 35-174 constitute the minimal region for p120 binding, or whether a shorter fragment is sufficient for the interaction.

Figure 3.2 Domain structure of Glis2. *Glis2 has 524 amino acids and contains a central Krüppel-like zinc finger domain with five zinc finger repeats (purple boxes). N-terminal to the zinc finger domain are the transcriptional activation and repressor domains. Amino acids 35-174 of Glis2 were shown to interact with full-length p120 in a yeast 2-hybrid screen.*

The zinc finger tandem domain of Glis2 shows high amino acid sequence identity with those of GLI family members (56-57%), while little homology was seen in the regions outside the zinc finger domains (Figure 1.7, Introduction; (Lamar et al., 2001; Zhang et al., 2002).

3.2.3 Testing reactivity of Glis2 antisera in different cell types

In order to analyse the role of endogenously expressed Glis2 in cell culture systems, two Glis2 antisera were raised in rabbit using GST-Glis2 (amino acids 35-174) as the antigen. These antibodies were not affinity purified. Total cell lysate from several cell lines were tested by Western blotting for expression of Glis2. Cell lysate from human embryonic kidney (HEK) 293 cells overexpressing FLAG-Glis2 was used as a positive control for Glis2 expression. Anti-Glis2 antiserum 1 showed good specificity for the Glis2 protein, whereas antiserum 2 detected the overexpressed protein but only very weakly detected endogenous Glis2 (Figure 3.3A).

Glis2 is endogenously expressed at detectable levels in HEK293, COS-1 (monkey fibroblast), and MCF7 (human breast adenocarcinoma) cells, but only weakly in Madine-Darby canine kidney (MDCK) cells using Glis2 antibody '1' (Figure 3.3A). Blotting with anti-FLAG antibody confirmed the expression of exogenous FLAG-Glis2 (Figure 3.3B). Therefore it seems that this rabbit polyclonal antiserum is able to pick up Glis2 expressed in human and monkey, but not canine cells. Glis2 antibody '1' was used to detect endogenous Glis2 protein in experiments described in this thesis.

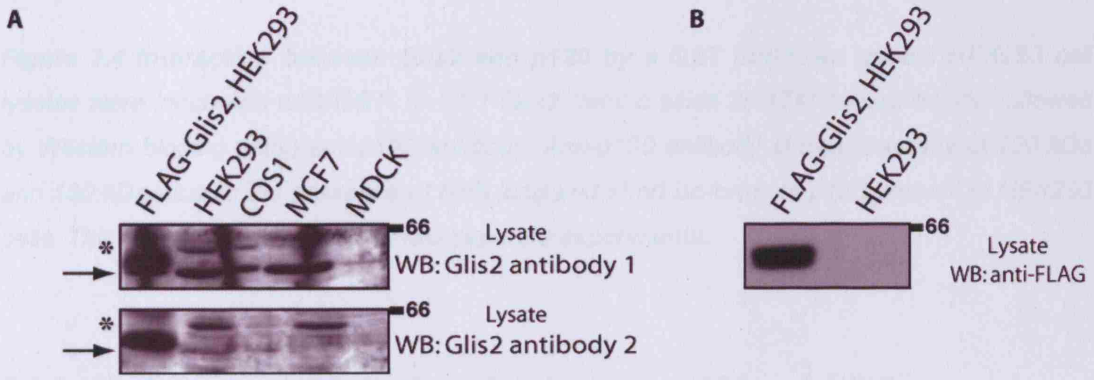

Figure 3.3 Testing reactivity of Glis2 antisera in a variety of cell lines. A *Cell lysates from HEK293, COS-1, MCF7 and MDCK cells were separated by polyacrylamide gel electrophoresis, followed by Western blotting using anti-Glis2 antibody '1' and antibody '2'. Glis2 antibody '1' showed good binding to the positive control, FLAG-Glis2 overexpressed in HEK293 cells, and endogenous Glis2 in HEK293, COS-1, MCF7, but only weakly in MDCK cells. Glis2 antibody '2' did not show good binding to endogenous Glis2. Endogenous Glis2 runs at about 56 kDa whereas FLAG-Glis2 migrates at about 60 kDa, due to the presence of the FLAG tag. Arrows indicate position of Glis2. Asterisks indicate position of what is most probably a non-specific band seen in HEK293 cells (both antibodies) and MCF7 cells (antibody '2').* ***B*** *Lysates of HEK293 cells expressing FLAG-Glis2 or empty vector were examined by Western blotting using anti-FLAG antibody.*

3.3 CHARACTERIZATION OF THE INTERACTION BETWEEN p120 AND GLIS2

3.3.1 Endogenous p120 binds to GST-Glis2 in a pulldown assay

To confirm the interaction between p120 and Glis2, a GST pulldown assay was performed. GST-Glis2 (amino acids 35-174) was produced by cloning the fragment of the Glis2 coding sequence isolated by the yeast 2-hybrid screen into a GST expression

vector (Y. Fujita). Endogenous p120 protein from HEK293 cell lysates interacted with GST-Glis2 (amino acids 35-174) beads, but not with GST beads (Figure 3.4).

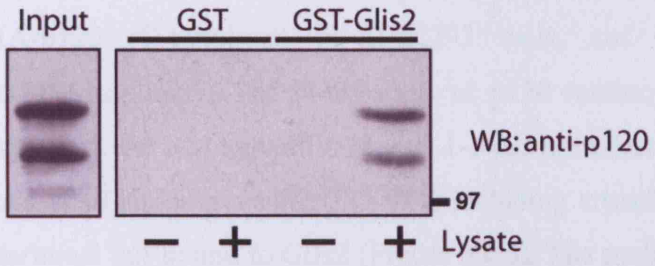

Figure 3.4 Interaction between Glis2 and p120 by a GST pulldown assay. *HEK293 cell lysates were incubated with GST- or GST-Glis2 (amino acids 35-174)-coated beads, followed by Western blotting using anti-p120 antibody. Anti-p120 antibody shows reactivity at 120 kDa and 100 kDa, due to the presence of both long and short isoforms of p120 present in HEK293 cells. This blot is representative of two separate experiments.*

3.3.2 Co-immunoprecipitation of endogenous p120 and Glis2

The interaction between p120 and Glis2 was further confirmed by immunoprecipitation of the two endogenous proteins in HEK293 cells. Endogenous Glis2 protein was co-immunoprecipitated with anti-p120 antibody, but not with control IgG (Figure 3.5).

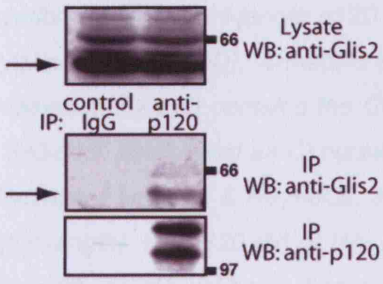

Figure 3.5 Interaction between Glis2 and p120 by immunoprecipitation*. HEK293 lysates were immunoprecipitated with control mouse IgG or anti-p120 antibody, and the precipitates were examined by Western blotting with anti-Glis2 and anti-p120 antibodies. Arrows indicate position of Glis2. This blot is representative of two separate experiments.*

3.3.3 Glis2 binds to the C-terminus of p120

To identify the Glis2-binding site on p120, truncation mutants of p120 were constructed (Figure 3.6A). FLAG-Glis2 was co-expressed with HA-p120, HA-p120 (N-terminus), or HA-p120 (C-terminus) in HEK293 cells, and lysates were precipitated using anti-HA antibody. The N-terminus of p120 (amino acids 1-647) including the amino-terminal end and armadillo repeats 1-6 did not interact with Glis2, whereas the C-terminus of p120 (amino acids 385-911) including armadillo repeats 2-10 and the carboxyl-terminal tail bound to Glis2 (Figure 3.6B). The mobility of the C-terminus of p120 (C) on SDS-PAGE was unexpectedly high, as it is expected to have a Molecular Weight of approximately 70 kDa. Sequencing of this construct confirmed the identity of the truncation mutant. This change in mobility could be due to structural changes resulting from the loss of the N-terminus.

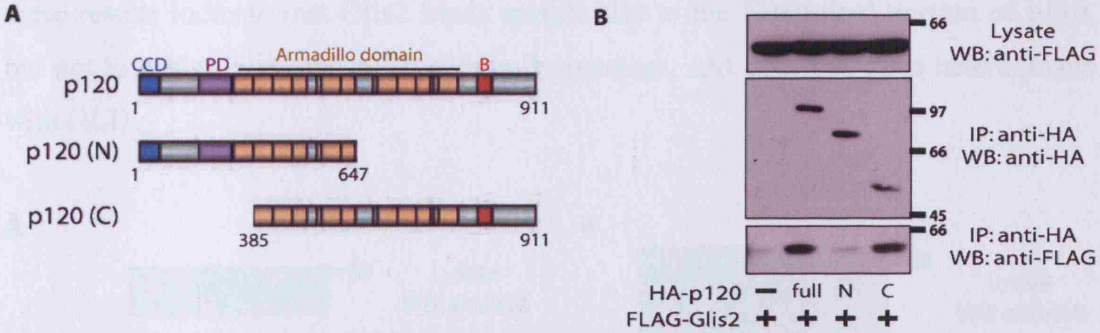

Figure 3.6 Interaction between Glis2 and p120 mutants by immunoprecipitation. A *Domain structure of p120 truncation mutants. Full-length p120 isoform 1B contains the coiled coil domain (CCD), phosphorylation domain (PD), Armadillo domain, and exon B. p120-N-terminus (amino acids 1-647: designated as N) contains the CCD, PD, and Arm repeats 1-6. p120-C-terminus (amino acids 385-911: designated as C) contains Arm repeats 2-10 and exon B. Schematic modified from Roczniak-Ferguson & Reynolds, J Cell Sci, 2003.* ***B*** *FLAG-Glis2 was expressed with HA-p120 (full length), HA-p120 (N) or HA- p120 (C) in HEK293 cells. Cell lysates were immunoprecipitated with anti-HA antibody, followed by Western blotting with anti-FLAG and anti-HA antibodies. This blot is representative of two separate experiments.*

3.3.4 Glis2 does not bind to GLI1 or δ-catenin

To further characterize the binding capabilities of Glis2, immunoprecipitation experiments were carried out using tagged Glis2 constructs expressed in HEK293 cells. It has been shown that GLI proteins can bind to members of another subfamily

of Krüppel-like transcription factors (named Zic) through their zinc finger domains (Koyabu et al., 2001). To test whether Glis2 can also bind to a GLI family member in an analogous fashion, HA-Glis2 was co-expressed with FLAG-GLI1 in HEK293 cells. Immunoprecipitation with anti-FLAG antibody did not pull down HA-Glis2 (Figure 3.7A).

Since Glis2 has been shown to be highly expressed in neuronal tissues (Lamar et al., 2001; Zhang et al., 2002), its ability to bind to δ-catenin was tested. δ-catenin (also known as NPRAP) is a p120 family member which is expressed at high levels in neuronal cells (Paffenholz and Franke, 1997). Overexpressing FLAG-δ-catenin or FLAG-p120 with HA-Glis2 and immunoprecipitating lysates with anti-FLAG antibody showed efficient binding of Glis2 to p120 but not δ-catenin (Figure 3.7B). This indicates the specificity of the interaction between Glis2 and p120. In summary, these results indicate that Glis2 binds specifically to the C-terminal portion of p120, but not to a highly related p120 subfamily member, and does not form heterodimers with GLI1.

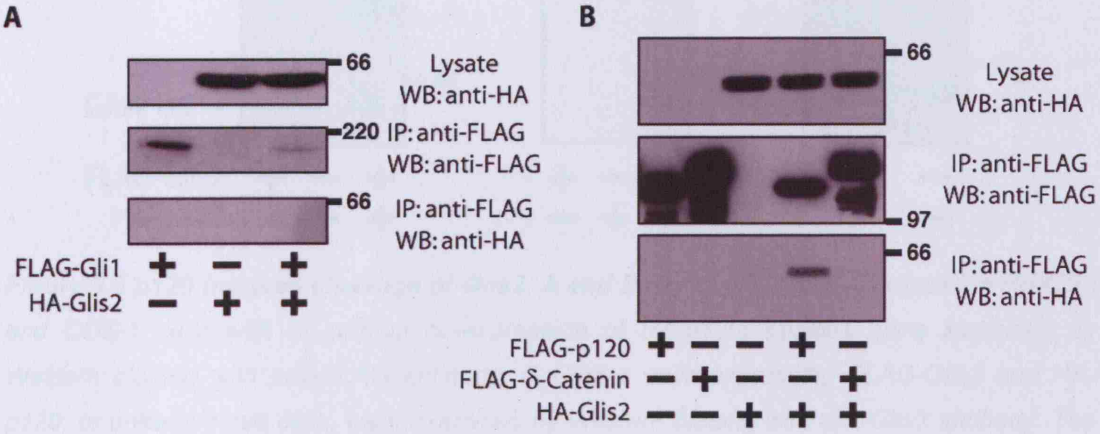

Figure 3.7 Glis2 does not bind to GLI1 or δ-catenin. A *Glis2 does not bind to GLI1. HEK293 cells were transiently transfected with FLAG-GLI1 and HA-Glis2. Cell lysates were immunoprecipitated using anti-FLAG antibody followed by Western blotting using anti-FLAG and anti-HA antibodies. These blots are representative of two separate experiments.* ***B*** *Glis2 does not bind to δ-catenin. HEK293 cells were transiently transfected with FLAG-p120 or FLAG-δ-catenin, and HA-Glis2. Cell lysates were immunoprecipitated with anti-FLAG antibody followed by Western blotting using anti-FLAG and anti-HA antibodies. These blots are representative of two separate experiments.*

3.4 CLEAVAGE OF GLIS2

3.4.1 p120-induced cleavage of Glis2

When HA-p120 and FLAG-Glis2 were co-expressed in HEK293 cells, it was discovered that cleavage of the Glis2 protein occurred, resulting in a product of approximately 40 kDa (Figure 3.8A). The FLAG tag is located at the N-terminus of Glis2, and since the cleavage product is detected using anti-FLAG antibody, this suggests that cleaved Glis2 loses a portion of its C-terminus. The cleavage product was therefore named Glis2 ΔC. A similar cleavage was also observed in COS-1 cells expressing FLAG-Glis2 and HA-p120 (Figure 3.8B), indicating that this cleavage is not cell type specific. As shown in Figure 3.8C, an approximately 40 kDa protein was detected in COS-1 cell lysates with anti-Glis2 antibody, which may indicate the presence of endogenous Glis2 ΔC.

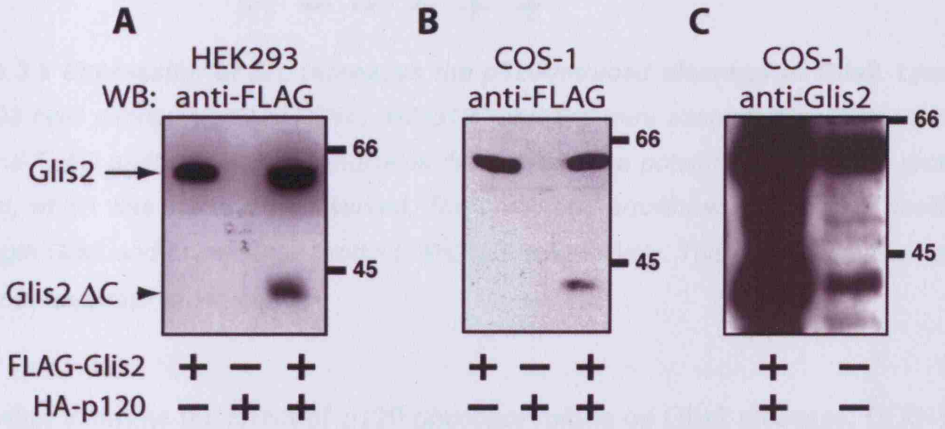

Figure 3.8 p120 induces cleavage of Glis2. A and B *FLAG-Glis2 was expressed in HEK293 and COS-1 cells with or without co-expression of HA-p120. Lysates were examined by Western blotting with anti-FLAG antibody.* ***C*** *COS-1 cells expressing FLAG-Glis2 and HA-p120, or untransfected cells, were examined by Western blotting with anti-Glis2 antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. These blots are representative of numerous separate experiments.*

3.4.2 Co-expression of Src increases p120-induced cleavage of Glis2

Since p120 was originally identified as a substrate of the tyrosine kinase Src (Reynolds et al., 1989), the effect of co-expression of Src on HA-p120-induced cleavage of FLAG-Glis2 was examined. Expression of Src alone did not induce Glis2 cleavage,

but co-expression of Src and p120 strongly enhanced the cleavage of Glis2 (Figure 3.9)[1]. Co-expression of p120 and Src also seems to stabilise the expression of full-length Glis2. These data suggest an additional role for tyrosine phosphorylation of p120, although it is not possible to rule out the phosphorylation of other components of the signalling pathway at this stage. It is important to examine the p120-induced cleavage of Glis2 in the presence of Src inhibitors, to investigate the mechanism of cleavage.

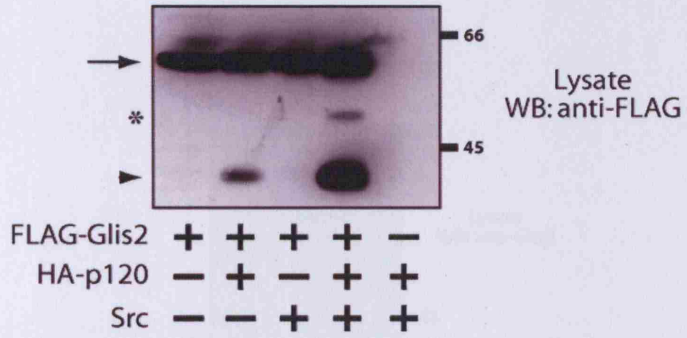

Figure 3.9 Expression of Src increases the p120-induced cleavage of Glis2. *Lysates of HEK293 cells expressing FLAG-Glis2, HA-p120 and Src were examined by Western blotting with anti-FLAG antibody. Asterisk indicates the position of a potential intermediate proteolytic product, which was sometimes observed. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blots is representative of numerous separate experiments.*

To further examine the effect of p120 phosphorylation on Glis2 cleavage, COS-1 cells were treated with Epidermal Growth Factor (EGF) and orthovanadate. p120 is known to be heavily tyrosine phosphorylated in response to EGF (Downing and Reynolds, 1991). Orthovanadate (VO_4^{3-}) inhibits protein tyrosine phosphatases and causes the accumulation of hyperphosphorylated proteins (Sakai, 1997). Treated cell lysates were examined by Western blotting using anti-Glis2 antibody. EGF-treated cells showed a slight increase in the amount of the 40 kDa product (Figure 3.10), whereas no cleavage of Glis2 was observed in the presence of orthovanadate (Figure 3.10). The greatly

[1] The amount of p120-induced cleavage of Glis2 was variable under different experimental conditions, however cells co-expressing HA-p120 and Src always exhibited substantial FLAG-Glis2 cleavage. Observations of HEK293 cells lysed and examined for Glis2 cleavage at various passage numbers and high or low confluence conditions did not yield further clues to the reason for the variations in cleavage levels.

reduced levels of both p120 and Glis2 in orthovanadate-treated cells, however, means that conclusions cannot be drawn from this part of the experiment. This is probably due to toxicity of orthovanadate treatment in COS-1 cells. The increase in the amount of Glis2 ΔC exhibited in the presence of EGF is supportive of a role of p120 tyrosine phosphorylation in inducing cleavage of Glis2. The results from this experiment were not followed up due to fact that the difference was minor, and a focus at the time on characterizing the identity of the cleavage product in HEK293 cells. Thus, firm conclusions cannot be drawn as to the extent of the effects of EGF and orthovanadate treatment.

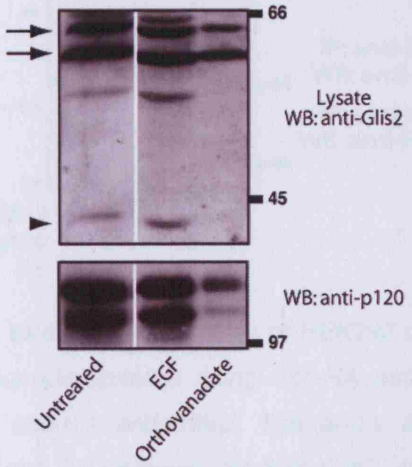

Figure 3.10 Effect of EGF and orthovanadate treatment on cleavage of Glis2. *COS-1 cells were treated with 100 ng/ml EGF for 4 hours, or with 500 nM orthovanadate for 2 hours. Total cell lysates were examined with anti-Glis2 and anti-p120 antibodies. The arrows and arrowhead indicate the positions of full-length Glis2 and the 40 kDa product, respectively. The double band for the endogenous full-length protein (double arrows) may be due to alternative splicing or protein degradation.*

3.4.3 p120 can bind to the cleaved form of Glis2 as well as full-length Glis2

To investigate whether p120 can still bind to the cleaved form of Glis2 as well as the full-length protein, a co-immunoprecipitation was performed between FLAG-Glis2 and HA-p120, expressed together with Src in HEK293 cells. The anti-HA antibody precipitated both FLAG-Glis2 and FLAG-Glis2 ΔC from cell lysates (Figure 3.11). This suggests that p120 may be involved in signalling downstream of the cleaved form of Glis2. As can be seen in Figure 3.11, the cleavage product of Glis2 is less detergent

soluble than the full length protein, as it exhibits much fainter reactivity when immunoprecipitated and detected by Western blot. This may indicate that the cleavage product is found in a complex with cellular components that are not solubilised by Triton X-100 detergent, indicating that other detergent conditions should be tested. However, since the presence of a 40 kDa Glis2 product in untransfected COS-1 cells (Figure 3.8) suggests that there is a physiological role for this protein product, and therefore it was considered relevant to investigate the cleavage product further in this study.

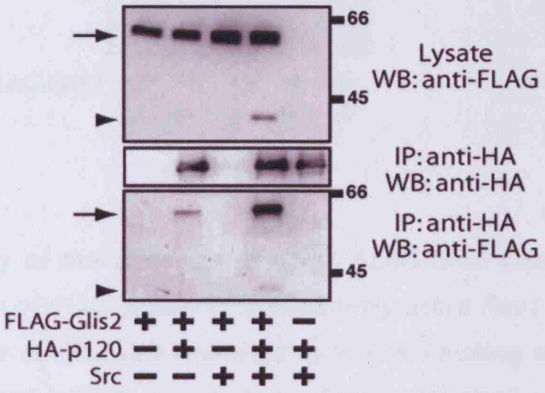

Figure 3.11 p120 also binds to Glis2 ΔC. *Lysates of HEK293 cells expressing FLAG-Glis2, HA-p120 and Src were immunoprecipitated using anti-HA antibody, followed by Western blotting with anti-FLAG and anti-HA antibodies. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

3.4.4 Neither β-catenin nor Rho GTPases induce cleavage of Glis2

To assess whether the induction of cleavage of Glis2 is specific to p120, another binding protein of the E-cadherin intracellular tail, β-catenin, was co-expressed in HEK293 cells with FLAG-tagged Glis2, but no cleavage of the Glis2 protein was observed (Figure 3.12). This suggests that other E-cadherin-binding proteins cannot substitute for p120 to cause Glis2 cleavage.

p120 is known to upregulate Rac and downregulate Rho activity (Anastasiadis et al., 2000; Noren et al., 2000). To mimic these downstream effects of p120, FLAG-Glis2 was co-expressed with constitutively active Rac1 G12V or inactive RhoA T17N, but no cleavage of Glis2 was observed under these conditions (Figure 3.12).

Together, these data with the immunoprecipitation results in Figures 3.5 and 3.11 suggest that it is the direct interaction between Glis2 and p120 that induces Glis2 cleavage.

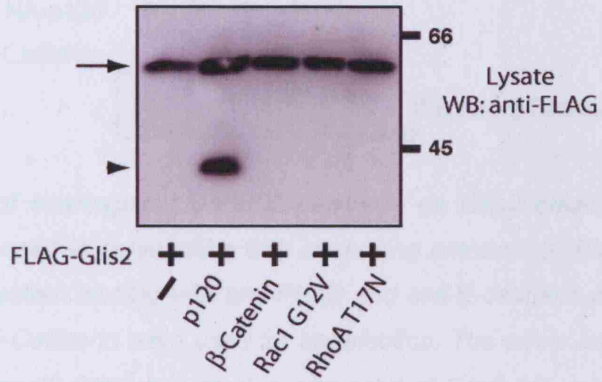

Figure 3.12 Specificity of the cleavage of Glis2. *FLAG-Glis2 was expressed in HEK293 cells with co-expression of p120, β-catenin, constitutively active Rac1 G12V or inactive RhoA T17N, and the cleavage of Glis2 was examined by Western blotting with anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

3.4.5 Increasing E-cadherin levels reduces p120-induced cleavage of Glis2

p120 is bound to cadherin at cell-cell contact sites. To investigate whether p120 can cause cleavage of Glis2 when it is bound to E-cadherin, FLAG-Glis2 was co-expressed with p120 and increasing amounts of E-cadherin in HEK293 cells. As the expression level of E-cadherin increased, the cleavage of Glis2 decreased (Figure 3.13), suggesting that p120 complexed with E-cadherin is not able to induce cleavage of Glis2.

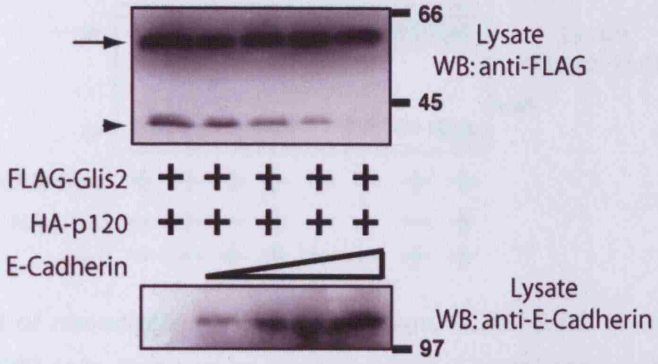

Figure 3.13 Effect of overexpression of E-cadherin on Glis2 cleavage. *FLAG-Glis2 and HA-p120 were expressed in conjunction with increasing amounts of E-cadherin, and lysates were analysed by Western blotting with anti-FLAG and anti-E-cadherin antibodies. 1, 2, 4 and 8 μg of pcDNA-HA-E-Cadherin were used for transfection. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

3.4.6 Nocodazole treatment increases p120-induced cleavage of Glis2

p120 has been shown to interact with microtubules (Chen et al., 2003; Franz and Ridley, 2004; Yanagisawa et al., 2004). The drug nocodazole prevents the polymerization of microtubules by sequestering tubulin monomers. The effect on Glis2 cleavage of releasing microtubule-bound p120 into the cytosol by nocodazole treatment was examined. In the presence of nocodazole, p120-induced cleavage of Glis2 was enhanced. Even in the absence of exogenous p120, nocodazole treatment together with Src induced the cleavage of Glis2 (Figure 3.14).

These data from the nocodazole and E-cadherin overexpression studies suggest that p120 must be free in the cytosol to interact with Glis2 and cause its cleavage. This is analogous to the interaction between p120 and its other nuclear binding partner, Kaiso, which also can only occur when p120 is free from sequestration by cadherins (Kelly et al., 2004; Roczniak-Ferguson and Reynolds, 2003).

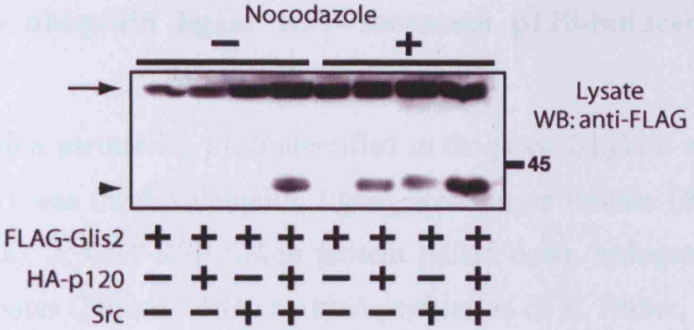

Figure 3.14 Effect of nocodazole on Glis2 cleavage. *FLAG-Glis2, HA-p120 and Src were expressed in HEK293 cells, followed by incubation with 5 μg/ml nocodazole for 4 h. Lysates were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

3.4.7 Ubiquitin increases p120-induced cleavage of Glis2

The cleavage of Glis2 is reminiscent of the C-terminal cleavage of GLI-family transcription factors. It has been shown that the cleavage of GLI involves the ubiquitination system (Jiang and Struhl, 1998). In order to address the contribution of ubiquitination on Glis2 cleavage, ubiquitin was overexpressed with FLAG-Glis2 and HA-p120 in HEK293 cells. Upon overexpression of ubiquitin, p120-induced cleavage of Glis2 was enhanced (Figure 3.15). This indicates that the mechanism of cleavage of Glis2 and GLI family members may be partly conserved.

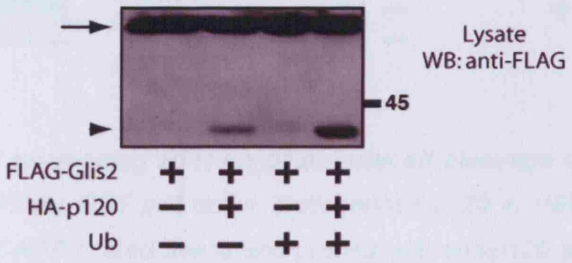

Figure 3.15 Effect of ubiquitin on Glis2 cleavage. *FLAG-Glis2, HA-p120 and HA-ubiquitin were expressed in HEK293 cells. Lysates were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

3.4.8 The E3 ubiquitin ligase RFP increases p120-induced cleavage of Glis2

Another interaction partner for p120 identified in the yeast 2-hybrid assay (described in Section 3.2.1) was the E3 ubiquitin ligase, Ret Finger Protein (RFP) (Y. Fujita, unpublished data). A GST-RFP fusion protein pulled down endogenous p120 from HEK293 cell lysates (Figure 3.16A, by kind permission of E. Ferber, Fujita Lab). To investigate whether this E3 ligase has an effect on p120-induced cleavage of Glis2, HA-RFP was co-expressed with FLAG-Glis2, HA-p120 and Src in HEK293 cells. Co-expression of RFP led to an increase in the p120-induced cleavage of Glis2, but RFP alone could not cause cleavage of Glis2 (Figure 3.16B). Co-expression of another E3 ubiquitin ligase Hakai, which binds to E-cadherin, was not able to affect p120-induced cleavage of Glis2 (Figure 3.16B). This suggests that RFP may induce ubiquitination of a component of the p120/Glis2 complex, and this may facilitate the cleavage of the Glis2 protein. Unfortunately, due to the poor quality of available anti-ubiquitin antibodies, it was not possible to fully determine the involvement of ubiquitination in the cleavage (data not shown).

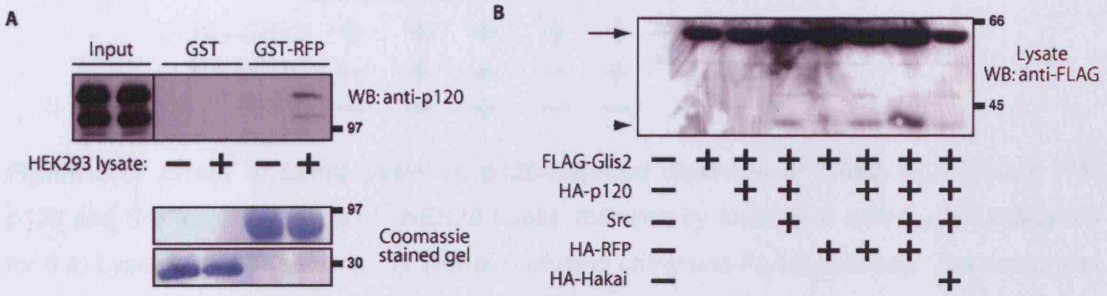

Figure 3.16 Effect of expressing RFP on p120-induced cleavage of Glis2. A *Interaction between p120 and RFP by GST pull down. Endogenous p120 in HEK293 cells was pulled down by GST- or GST-RFP-coated beads and probed with anti-p120 antibody. Lower panels show sections of the gel stained with Coomassie Blue to indicate the expression of GST (30 kDa) and GST-RFP (approximately 90 kDa). By kind permission of E. Ferber.* ***B*** *Expression of HA-RFP enhances p120-induced cleavage of Glis2. Lysates of HEK293 cells expressing FLAG-Glis2, HA-p120, Src and the E3 ubiquitin ligases RFP and Hakai, were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

3.4.9 Lactacystin has no effect on p120-induced cleavage of Glis2

Proteins that are subject to proteolytic cleavage as a result of ubiquitination are processed by the proteasome (reviewed in (Nandi et al., 2006). To investigate further the contribution of ubiquitination on Glis2 cleavage, the proteasome inhibitor Lactacystin was used to treat HEK293 cells expressing FLAG-Glis2, HA-p120 and Src. It was expected that if the proteolytic machinery was inhibited, the cleavage of Glis2 would decrease in treated cells. However, no decrease in cleavage was observed (Figure 3.17). These data suggest that other proteases may be involved or that if Glis2 does gain a ubiquitin 'tag', it acts as a signalling moiety rather than a direct signal for proteolytic processing (Haglund and Dikic, 2005).

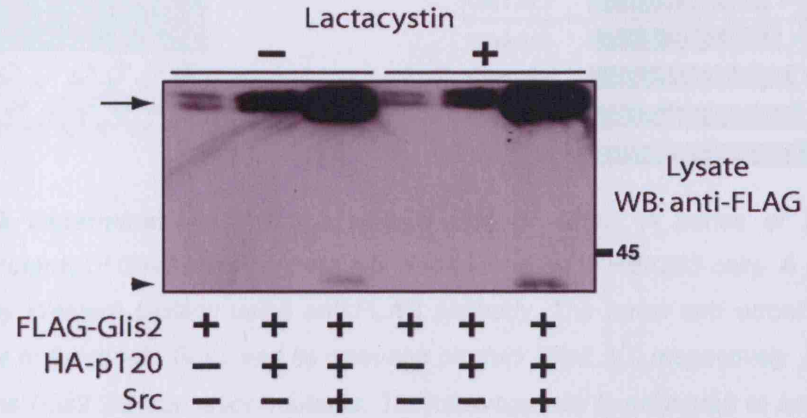

Figure 3.17 Effect of Lactacystin on p120-induced cleavage of Glis2. *FLAG-Glis2, HA-p120 and Src were expressed in HEK293 cells, followed by incubation with 5 μM Lactacystin for 6 h. Lysates were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments. Stability of the full-length Glis2 protein is increased by the co-expression of p120 and Src in this experiment.*

3.4.10 Constructing FLAG-tagged Glis2 ΔC

In order to analyse the role of the cleaved form of Glis2, attempts were made to determine the C-terminal amino acid sequence of Glis2 ΔC by mass spectrometry. However, these attempts were unsuccessful due to the low expression level of Glis2 and low detergent solubility of the cleaved form. As an alternative, a series of FLAG-tagged C-terminal truncation mutants of Glis2 were constructed by PCR (See Table

2.2, Materials and Methods), which were named Glis2 ΔC1-6 (Figure 3.18). These truncation mutants were expressed in HEK293 cells and the sizes of the mutants were compared with that of FLAG-Glis2 ΔC by Western blotting. As shown in Figure 3.18A, FLAG-Glis2 ΔC exhibited the same mobility in SDS-PAGE as FLAG-Glis2 ΔC2 (amino acids 1-290). The C-terminus of Glis2 ΔC2 terminates at the second histidine residue of the fourth zinc finger domain. Therefore, it is most likely that p120-induced cleavage of Glis2 results in the loss of the last zinc finger domain to yield Glis2 ΔC (Figure 3.18B).

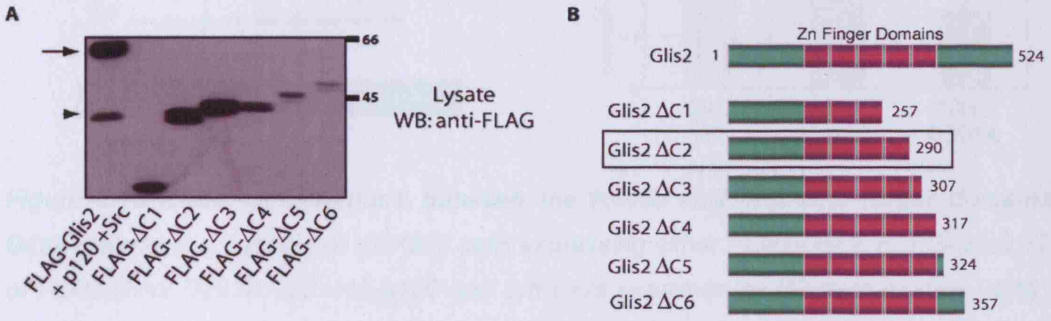

Figure 3.18 Determination of the cleavage site of Glis2. *A series of FLAG-tagged truncation mutants of Glis2 were constructed, and expressed in HEK293 cells.* ***A*** *Lysates were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively.* ***B*** *Diagram of Glis2 and the Glis2 ΔC truncation mutants. The cleavage site is estimated to be directly after the fourth zinc finger domain, equivalent to Glis2 ΔC2 (boxed).*

3.4.11 Mutation of Glis2 residue Glutamate 293 affects p120-induced cleavage whereas mutation of residue Tyrosine 291 does not

The linker region between the fourth and fifth zinc finger domains of Glis2 was studied to find candidate amino acids that may play a role in the cleavage of Glis2. Tyrosine(Y)291 and Aspartic acid(D)293 are close to the putative cleavage site (as determined by mutational analysis, Section 3.4.10). Since it is possible that tyrosine phosphorylation of Y291, or steric interaction of the charged side chain of D293, could affect the interaction of the proteolytic machinery with Glis2, each of these residues were mutated to alanine to examine the effect on p120-induced cleavage of Glis2. The Glis2 Y291A mutation did not significantly affect the cleavage of Glis2, whereas the Glis2 D293A mutation exhibited a 25% reduction in cleavage induced by p120 and Src

compared with wild type Glis2 (Figure 3.19). These data suggest that the linker region between the fourth and fifth zinc finger domain could indeed be involved in the interaction with the proteolysis machinery, although the possibility of Y291 phosphorylation does not seem to be important.

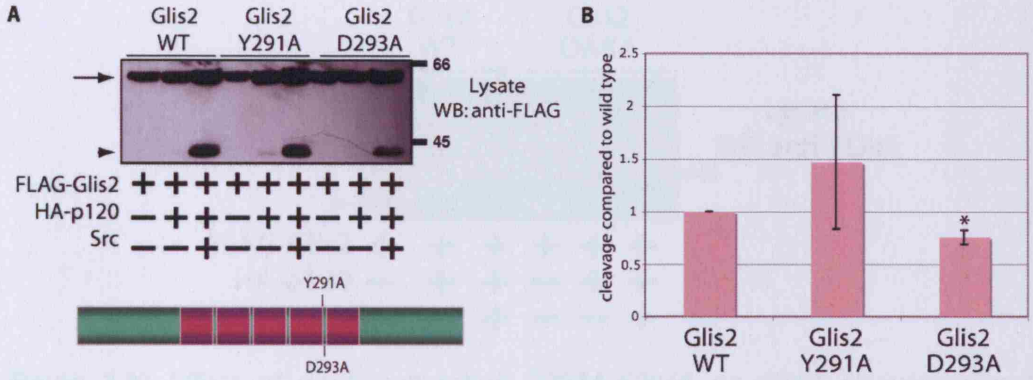

Figure 3.19 Effect of mutations between the fourth and fifth zinc finger domains on Glis2 cleavage. A *Lysates of HEK293 cells expressing either FLAG-Glis2, FLAG-Glis2 Y291A or FLAG-Glis2 D293A, with HA-p120 and Src were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.* ***B*** *The band intensities of the full-length and cleaved proteins were analysed by densitometry. The percentage of Glis2 cleavage observed in the wild type protein was normalised to 1 and the levels of Glis2 cleavage in the mutants were compared to wild type. *, approaching significance for lower cleavage than wild type Glis2 (p value = 0.06).*

3.4.12 Glis2 mutant D293A-K294A does not affect p120-induced cleavage

Further examination of the linker region between the fourth and fifth zinc finger domains of Glis2 showed that the residue next to D293 is a lysine (K294). Proteins are ubiquitinated on lysine residues (reviewed in (Haglund and Dikic, 2005)). Since there is some evidence to suggest that ubiquitination plays a role in Glis2 cleavage (Figure 3.15), the role of residue K294 was investigated to determine if it is also involved in the cleavage process. A double mutant D293A-K294A (DAKA) was constructed and co-expressed with p120 and Src in HEK293 cells. However, no decrease in the cleavage of Glis2 was observed (Figure 3.20), in contrast to the result for the D293A mutant. This suggests that ubiquitination of this lysine residue is not critical to the cleavage process. These data also indicate that there may be a requirement for a

specific charge ratio in the amino acids in the linker region, since mutating an aspartic acid (negatively charged) residue alone has an effect but mutating both this and lysine (positively charged) amino acid suppresses the decrease in cleavage seen for the D293A mutant. In order to confirm this, both mutants should be compared side to side on the same blot.

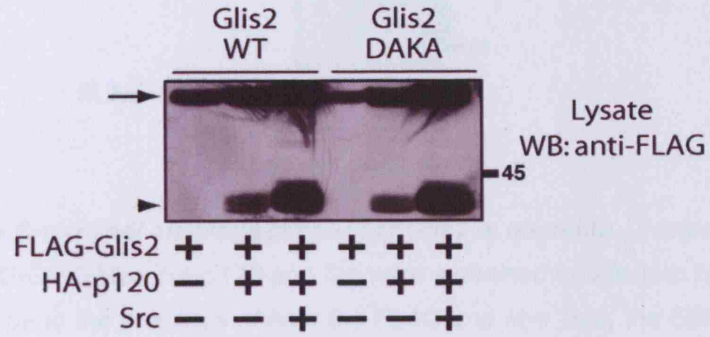

Figure 3.20 Effect of double mutation D293A-K294A on Glis2 cleavage. *Lysates of HEK293 cells expressing either FLAG-Glis2 or FLAG-Glis2 D293A-K294A (DAKA), with HA-p120 and Src were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively.*

3.4.13 The C-terminal cleavage product of Glis2 is highly unstable

The fate of the C-terminal cleavage product of Glis2 was examined, by making a C-terminally tagged construct, FLAG-Glis2-Myc. This construct was co-expressed with HA-p120 and Src in HEK293 cells and cell lysates were blotted with anti-Myc antibody (Figure 3.21). This showed that the C-terminus of Glis2 is highly unstable, as a smear of reactivity is detected with anti-Myc antibody, suggesting the formation of degradation intermediates.

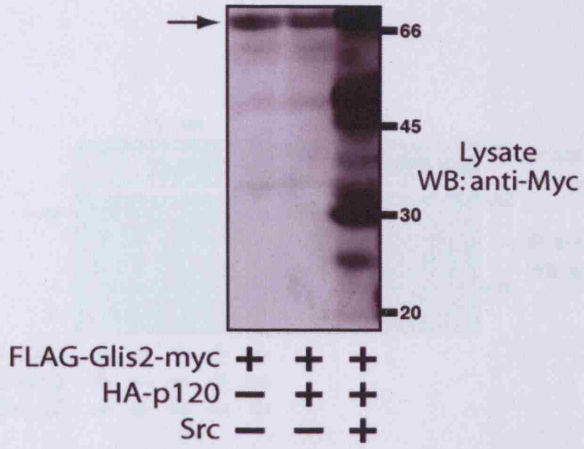

Figure 3.21 The C-terminal cleavage product of Glis2 is unstable. *Lysates of HEK293 cells expressing FLAG-Glis2-Myc, HA-p120 and Src were examined by Western blotting using anti-Myc antibody. Due to the presence of both the FLAG and Myc tags, the Glis2 protein runs at approximately 68 kDa. The arrow indicates the position of Glis2. This blot is representative of two separate experiments.*

3.4.14 Glis2 does not homodimerise

Some zinc-finger domain transcription factors, such as Kaiso, can form homodimers (Daniel and Reynolds, 1999). To test whether Glis2 can form homodimers or full-length/cleaved heterodimers, experiments using FLAG-tagged and HA-tagged constructs of Glis2 and FLAG-tagged Glis2 ΔC were carried out. Co-expression of FLAG-Glis2 and HA-Glis2 and subsequent immunoprecipitation using anti-FLAG antibody did not show any binding of HA-Glis2 in the precipitates (Figure 3.22). This indicates that Glis2 does not homodimerise. In addition, co-expression of FLAG-Glis2 ΔC and HA-Glis2 and subsequent immunoprecipitation with anti-FLAG antibody also showed no interaction, indicating that Glis2 does not bind to Glis2 ΔC.

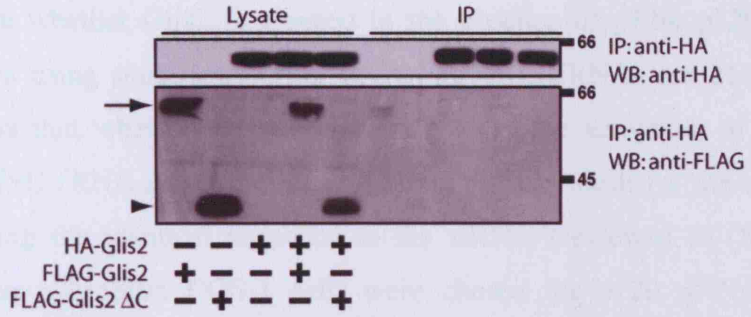

Figure 3.22 Glis2 does not homodimerise or form heterodimers with Glis2 ΔC. *HEK293 cells expressing HA-Glis2 with either FLAG-Glis2 or FLAG-Glis2 ΔC were immunoprecipitated using anti-HA antibody, followed by Western blotting with anti-HA and anti-FLAG antibodies. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. These blots are representative of two separate experiments.*

3.5 RNAi KNOCKDOWN OF p120

To investigate whether Glis2 is cleaved in the absence of p120, p120 protein was knocked down using small interfering RNA (siRNA). siRNAs are 21-23 nucleotide RNA products that when expressed in cells lead to the activation of the silencing machinery RISC (RNA-induced silencing complex) that destroys the mRNA of the gene containing the identical sequence to the siRNA (reviewed in (Sen and Blau, 2006). Monkey fibroblast COS-1 cells were chosen for p120 siRNA knockdown treatment because the endogenously expressed Glis2 in these cells exhibits a potential cleavage product that may be affected by p120 loss (Figure 3.8C).

Two methods were tried: a vector based system, in order to create stable cell lines of clones lacking p120 expression; and a transient knockdown system using siRNA oligonucleotides (oligos). Despite initial success using the vector based system for p120 knockdown (siRNA oligo sequences shown in Table 2.6, Section 2.1.11, Materials and Methods), attempts to select for colonies of RNAi positive (p120 negative) cells were unsuccessful, as no clones survived under the selection conditions (even though optimal selective conditions were determined). Therefore I used the transient transfection system as an alternative.

3.5.1 Knockdown of p120 using siRNA oligonucleotides

Using the online tool on the Qiagen website, two siRNA oligonucleotides (oligos) were designed (Table 2.7, Section 2.1.11, Materials and Methods), using the criteria that the oligos would knock down every isoform of p120 in both monkey and human cells (to keep open the possibility of using the p120 siRNA oligos in human cell lines). COS-1 cells were transiently transfected with these two siRNA oligos using Hi-PerFect® reagent, and knockdown was monitored by Western blotting of cell lysates using anti-p120 antibody. Knockdown of p120 levels by over 90% was achieved at 96 hours post-transfection using 1.5 μg of both p120 siRNAs compared to cells transfected with 1.5 μg of a control non-silencing oligo (Figure 3.23A and B). A control MAPK siRNA oligo also showed effective knockdown when lysates were probed with anti-MAPK antibody (Figure 3.23A), which shows that the system was working according to the manufacturers instructions. To check equal protein loading,

samples were blotted with anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody. The slight difference in the levels of GAPDH present in each sample does not account for the significant knockdown seen for p120 levels (Figure 3.23C).

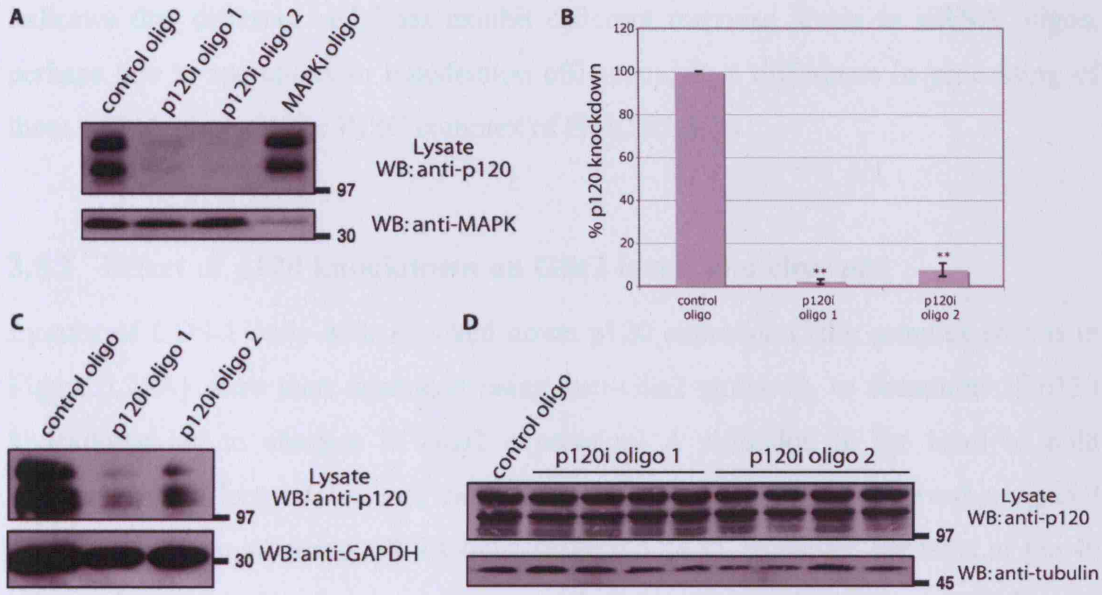

Figure 3.23 Knockdown of p120 using siRNA oligos. A *p120 is knocked down specifically. COS-1 cells were transiently transfected with a control non-silencing siRNA, two siRNAs for p120, and a positive control RNAi oligo for MAPK. Lysates were analysed at 96 h after transfection and examined by Western blotting using anti-p120 and anti-MAPK antibodies. Equal protein loading was achieved using a BioRad protein concentration ELISA kit. These blot are representative of two separate experiments.* ***B*** *The band intensities from the anti-p120 Western blots were analysed by densitometry. Protein expression levels in control siRNA-treated cells were normalised to 100%. **, significantly lower than control cells (p values < 0.001).* ***C*** *Protein loading does not account for knockdown of p120 expression. COS-1 cells were transiently transfected with a control non-silencing RNAi oligo and two RNAi oligos for p120. Lysates were examined by Western blotting using anti-p120 and anti-GAPDH antibodies.* ***D*** *p120 is not knocked down in HEK293 cells. HEK293 cells were transiently transfected with a control non-silencing RNAi oligo, and two RNAi oligos for p120. Each p120 RNAi oligo was transfected at two amounts (1 μg and 1.5 μg) and cells were incubated for two timepoints (72 h and 96 h). Lysates were examined by Western blotting using anti-p120 and anti-tubulin antibodies.*

Using the p120i oligos in HEK293 cells, however, did not result in p120 knockdown, despite the sequence of the human p120 being identical to that contained within the siRNAs (Figure 3.23D). Two time points were taken (72 h and 96 h) and two amounts of siRNA (1 μg and 1.5 μg) were transfected at each time point. Equal protein loading in this experiment was determined by blotting with anti-tubulin (Figure 3.23D). This indicates that different cell lines exhibit different response levels to siRNA oligos, perhaps due to variations in transfection efficiency, or a difference in processing of these siRNA oligos in the RISC complex of HEK293 cells.

3.5.2 Effect of p120 knockdown on Glis2 levels and cleavage

Lysates of COS-1 cells with knocked down p120 expression (the samples shown in Figure 3.23A) were then examined using anti-Glis2 antibody, to determine if p120 knockdown led to changes in Glis2 expression. A reduction in the level of both endogenous full-length Glis2 and the 40 kDa cleavage product was observed compared to the control non-silencing siRNA oligo (Figure 3.24A). However, the level of the 40 kDa product was reduced to a greater extent than that of the full-length protein. Protein levels of full-length Glis2 in this experiment fell by approximately 20% compared with over a 40% reduction in the proportion of the 40 kDa product present in p120 siRNA oligo '1'-treated cells, as determined by densitometric analysis (Figure 3.24B). These data suggest that p120 stabilizes the Glis2 protein and that p120 is involved in the processing of Glis2, thus supporting the overexpression data presented in this chapter.

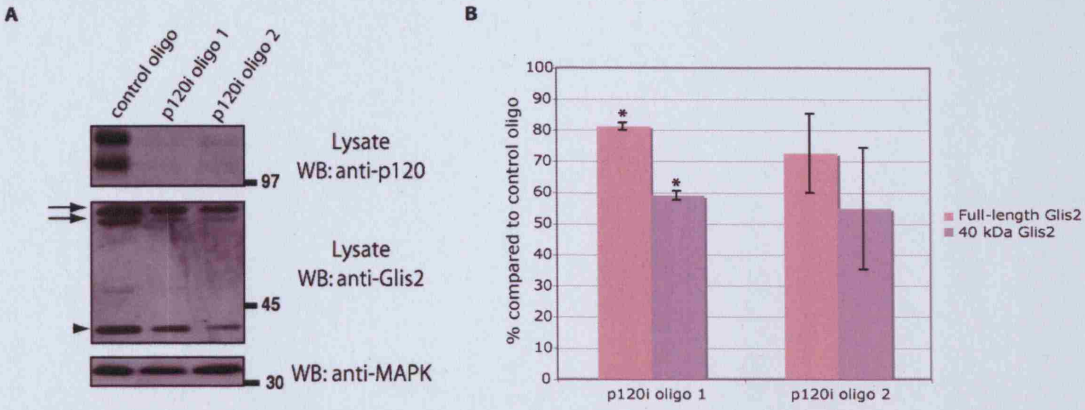

Figure 3.24 Effect of p120 RNAi on Glis2. A *COS-1 cells were transiently transfected with a control non-silencing RNAi oligo or two RNAi oligos for p120. After 96 h, cell lysates were examined by Western blotting using anti-p120 and anti-Glis2 antibodies. Equal protein loading was confirmed by Western blotting using anti-MAPK antibody. The arrows and arrowhead indicate the positions of full-length Glis2 and the 40 kDa product, respectively. The double band for the endogenous full-length protein (arrows) may be due to alternative splicing or protein degradation. These blots are representative of two separate experiments.* ***B*** *The band intensities of full-length Glis2 and the 40 kDa product were analysed by densitometry. The level of Glis2 present in control siRNA-treated cells was normalised to 100%. The proportion of 40 kDa Glis2 present in control siRNA-treated cells was also normalised to 100% to allow comparison between two different experiments. *, significantly lower than control siRNA-treated cells (p values < 0.01).*

3.6 DISCUSSION

This chapter describes the identification of a novel binding partner for p120, the transcriptional repressor Glis2, and the characterization of the resultant cleavage of Glis2 in the presence of p120. The discovery of a second transcription factor (in addition to Kaiso) that binds to p120 provides strong evidence to suggest that p120 has functions in the nucleus, and points to additional signalling functions for the adherens junction, linking events at the cell surface with transcriptional regulation in the nucleus. The observations of p120-induced cleavage of Glis2 suggest that the truncated form of Glis2 may be important for signalling downstream of the p120/Glis2 interaction.

Glis2 binds to p120 through a binding site N-terminal to its zinc finger tandem repeat domain, a region slightly overlapping with the transcriptional regulation domains of Glis2 (Figure 3.2). The C-terminal portion of p120 is required for Glis2 binding. Whether Glis2 is still able to affect transcription when complexed to p120 is investigated in Chapter 4. Glis2 is the second identified nuclear binding protein of p120, after Kaiso (Daniel and Reynolds, 1999). Both Kaiso and Glis2 are zinc finger-containing transcription factors with repressor activity (Prokhortchouk et al., 2001; Zhang et al., 2002). For p120 to interact with either of its nuclear binding partners, it must be free from sequestration by E-cadherin and/or microtubules. However, in contrast, no processing of Kaiso upon p120 binding has been described. The stimuli that induce p120 binding to either of its nuclear binding partners are unknown.

Glis2 is homologous to the Ci/GLI family of transcription factors (Figure 1.7, Introduction). Functional activity of Ci/GLI is regulated by C-terminal cleavage, whereby a signal propagated by Shh binding to its receptor leads to the processing of the full-length transcriptional repressor form of Ci/GLI into the short transcriptional activator form (Section 1.4, Introduction; (Osterlund and Kogerman, 2006). In an analogous way, Glis2 exhibits C-terminal cleavage that is induced by p120 and Src co-expression, which suggests that Glis2 transcriptional regulation may also be regulated by proteolytic processing. Another similarity between the two systems is that the ubiquitination machinery seems to be involved in cleavage of both Glis2 and GLI family members (Jiang and Struhl, 1998). However, the mode of cleavage differs

between the two systems. Cleavage probably occurs between the fourth and fifth zinc finger domains in Glis2, whereas for GLI the cleavage site is outside the zinc finger tandem region. Furthermore, Glis2 cleavage is enhanced by tyrosine kinase Src, while serine/threonine kinases, such as GSK-3β, PKA and casein kinase 1, are involved in the cleavage of GLI (Ingham and McMahon, 2001; Price and Kalderon, 2002).

The potential involvement of Src in directing transcriptional regulation downstream of p120 is very interesting for two reasons. First, the role of tyrosine phosphorylation of p120 is still rather obscure (described in Section 1.3.1) and this evidence of phosphorylation promoting the interaction between p120 and Glis2 and resultant Glis2 cleavage provides a new explanation for this modification. However, since it is the C-terminal portion of p120 that contains the Glis2 binding site, this rules out the possibility that tyrosine phosphorylation of p120 in its N-terminal regulatory domain (Figure 1.4, Introduction) creates a binding site for Glis2. Perhaps instead phosphorylation of p120 by Src leads to enhanced recruitment of another factor important for Glis2 cleavage, for example RFP[2]. The second reason that involvement of Src in enhancing p120-induced cleavage of Glis2 is of interest is that Src has long been known to play a role in human tumour progression, but the mechanisms involved are quite unclear (Frame, 2002). If Src's role in tumour formation included altered transcription due to aberrant signalling through p120/Glis2, this would extend its role from causing morphological changes in cancerous cells to include gene regulation downstream of junctional complex signalling.

Co-expression of p120 and Src also seems to increase the stability of Glis2 protein levels because detection of Glis2 by anti-FLAG antibody in Western blots is much greater in lysates transfected with all three proteins (particularly clear examples in Figures 3.14 and 3.17). This is confirmed by the observation that p120 siRNA-treated COS-1 cells exhibit a 20% reduction in the level of Glis2 protein, compared to control siRNA-treated cells (Figure 3.25). The reason for this may be that binding to p120 protects Glis2 from degradation, while simultaneously stimulating its specific C-terminal cleavage.

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[2] Attempts were made to investigate the interaction between p120 and RFP in the presence of Src by immunoprecipitation, but due to the detergent insoluble nature of RFP, this was not possible.

The fact that the cleaved form of Glis2 loses one of its five zinc fingers in the tandem repeat domain raises some interesting questions. Whether Glis2 ΔC can still bind to DNA is the most pressing issue, since the hypothesis presented above - where p120 can direct an alternative course of transcriptional regulation by causing Glis2 cleavage - rests on the ability of Glis2 ΔC to remain competent to affect transcription. Glis2 ΔC could potentially bind to different sites on DNA to the full-length protein, or alternatively, it could compete for binding with full-length Glis2. It is also possible that the C-terminal cleavage fragment of Glis2 containing the fifth zinc finger domain could have signalling or transcriptional regulation functions, but due to the instability of this protein product (Figure 3.21), this was not further examined. Indeed, the fate of the C-terminal fragment of Ci/GLI has not been reported either. A construct expressing the N-terminal cleaved form of Glis2 was produced (Figure 3.18), which was used as a tool to address the function of Glis2 and Glis2 ΔC in the nucleus, and this is described in Chapter 4.

A drawback of the data presented here is that experiments were not conducted in epithelial cells with well established adherens junctions. HEK 293 cells are only loosely associated and so endogenous p120 is not tightly associated with AJs, meaning that the relevance of this data to physiological epithelial cell systems is not known. An experimental system using MCF epithelial cells would give more information about the importance of AJ integrity to the p120/Glis2 interaction, as invasive MCF7 cells with low AJ integrity could be compared with non-invasive MCF10 cells with high AJ integrity. Blotting for Glis2 in these cell lines would show if differences in AJ integrity has an effect on the cleavage of Glis2, or the interaction of p120 with Glis2.

CHAPTER 4

GLIS2 AND p120 FUNCTION

IN THE NUCLEUS

4.1 INTRODUCTION

To address the significance of the interaction between p120 and Glis2, functional studies were undertaken. This association with a transcription factor indicates a possible new role for p120 in the nucleus, perhaps influencing the switching on and off of Glis2 target genes. Indeed, it has been shown recently that p120 binding to the transcriptional repressor Kaiso prevents Kaiso from binding to target sequences of DNA (Kelly et al., 2004; Kim et al., 2004; Park et al., 2005).

In this chapter, the cellular localisation of p120 in the presence of Glis2 is examined. Translocation of p120 into the nucleus has been observed in a number of cell lines, and this suggests that p120 is required in the nucleus to fulfil a specific function. Here, data are presented to show that in the presence of Glis2 and Src, p120 is translocated into the nucleus, suggesting that the functional role of p120 in the nucleus may be related to its interaction with Glis2 (Section 4.2). This directed nuclear translocation of p120 upon the expression of an interaction partner has not been described before, even for Kaiso (A. Reynolds, personal communication).

Next, the ability of Glis2 and Glis2 ΔC to bind to DNA in the presence or absence of p120 and Src are examined. The indication here that Glis2 ΔC can bind DNA suggests that this shorter form of the transcription factor may also play a role in transcriptional regulation (Section 4.3). *In vivo* studies in the chick neural tube show that Glis2 can inhibit neuronal differentiation, and moreover, the results suggest that Glis2 ΔC together with p120 can affect neuronal differentiation to the same extent as full-length Glis2 alone (Section 4.4). Dorso-ventral patterning in the neural tube was also examined as this is affected in GLI overexpression studies, and Glis2 has been shown to bind to the GLI binding site on DNA. However, no clear involvement of Glis2 or Glis2 ΔC in dorso-ventral patterning was shown in this system (Section 4.4).

4.2 LOCALIZATION OF p120 AND GLIS2

4.2.1 p120 localizes to the nucleus in the presence of Glis2 and Src

HA-p120 was expressed in HEK293 cells and its localisation was examined by immunofluorescence using anti-HA antibody. p120 primarily localized to the plasma membrane with diffuse cytoplasmic staining (Figure 4.1A). When Src was co-expressed with HA-p120, no change was seen in the distribution of p120 (Figure 4.1A). When FLAG-Glis2 was co-expressed with HA-p120, again the p120 staining was seen primarily at the plasma membrane with a small proportion seen in the nucleus, however nuclear translocation was not consistently observed under these conditions (Figure 4.1A). However, when FLAG-Glis2 and Src together were co-expressed with HA-p120, a marked redistribution of p120 into the nucleus was observed (Figure 4.1A). Of cells transfected with HA-p120, nearly all of them (approximately 90%) could be seen to exhibit nuclear staining (This effect was not quantified statistically). A similar effect was also observed in COS-1 cells (by kind permission of Y. Fujita; Figure 4.1B).

Next, endogenous p120 localisation was examined using mouse fibroblast L cells. L cells do not express classical cadherins, leading to the accumulation of a cytoplasmic pool of p120 (Ohkubo and Ozawa, 1999). Overexpression of Glis2 induced nuclear translocation of endogenous p120 (Figure 4.2A). Expression of Src also showed a similar but weaker effect (Figure 4.2B). Co-expression of Glis2 and Src further augmented the nuclear translocation of endogenous p120 (Figure 4.2B) (p value for Glis2 vs. Glis2+Src = 0.03). Quantification of the data was performed by measuring the pixel intensity of a defined region (a circle of 1.5 μm diameter) inside the nucleus and in the cytoplasm, to determine the ratio of p120 localisation.

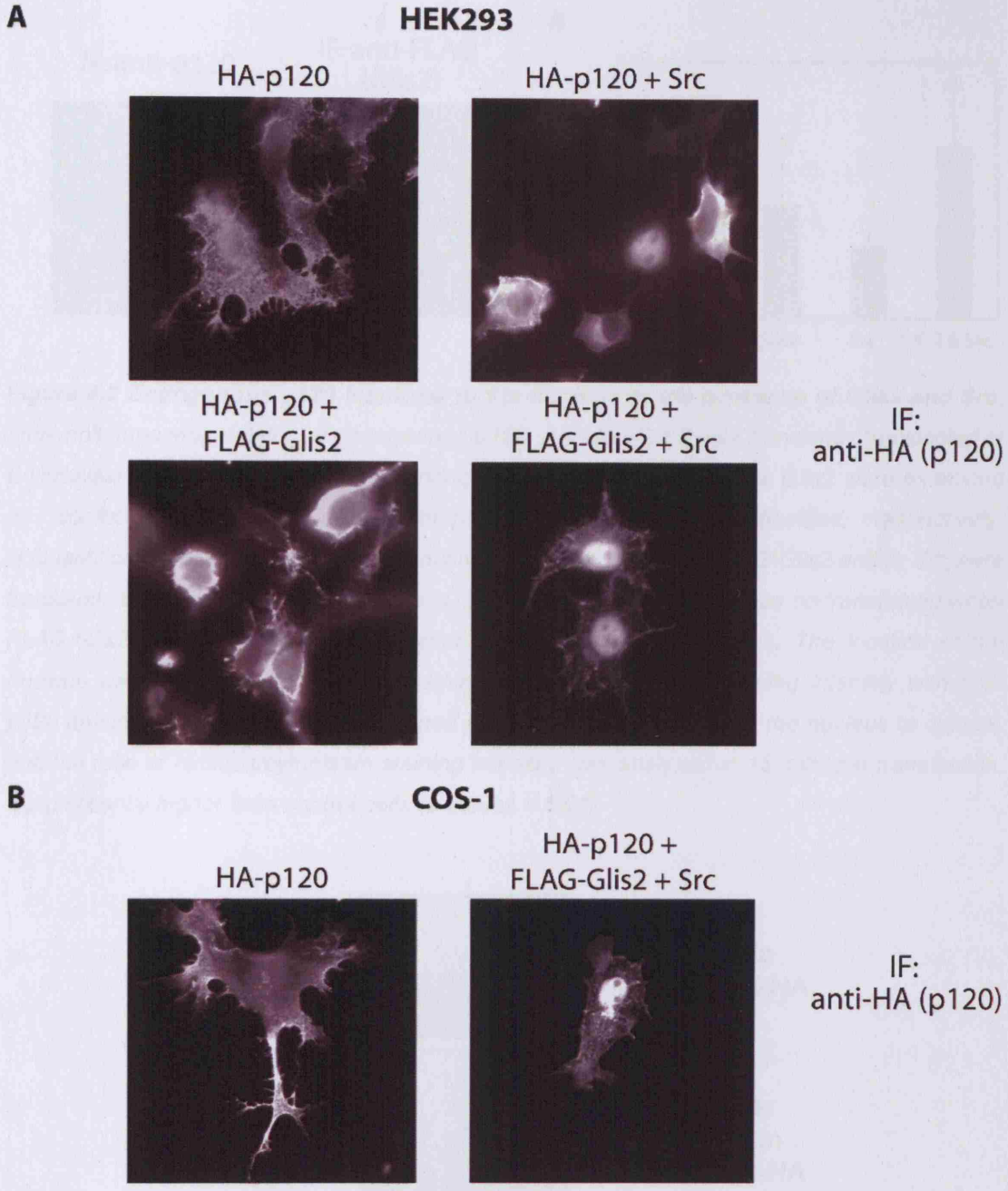

Figure 4.1 p120 localizes to the nucleus in the presence of Glis2 and Src. *Immunofluorescent staining of exogenous p120. HA-p120 was transiently transfected in cells with or without co-expression of FLAG-Glis2 and Src. The localization of p120 was examined by epifluorescent microscopy using anti-HA antibody.* ***A*** *HEK293 cells.* ***B*** *COS-1 cells.*

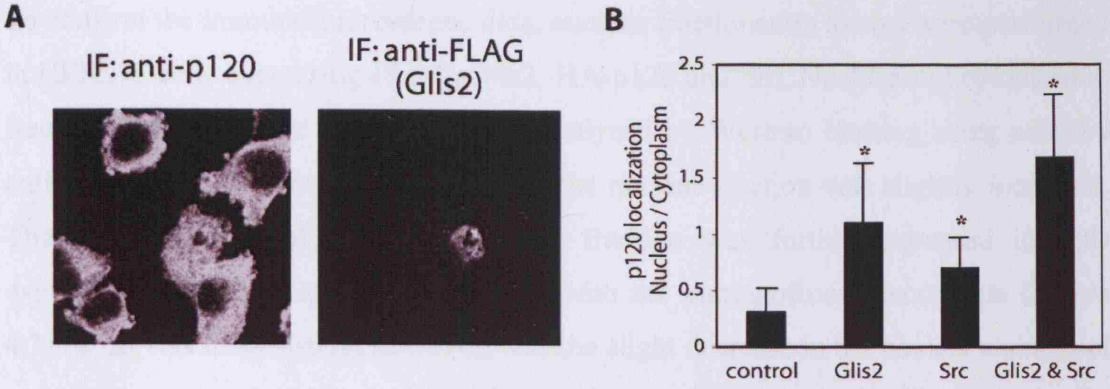

Figure 4.2 Endogenous p120 localizes to the nucleus in the presence of Glis2 and Src. *Immunofluorescent staining of endogenous p120.* ***A*** *FLAG-Glis2 was transiently transfected in L fibroblast cells. The localization of endogenous p120 and exogenous Glis2 were examined by confocal microscopy using anti-p120 and anti-FLAG antibodies, respectively.* ***B*** *Quantification of the nuclear translocation of endogenous p120. FLAG-Glis2 and/or Src were transiently transfected in L fibroblast cells. The empty pEGFP vector was co-transfected when FLAG-Glis2 was not transfected in order to identify transfected cells. The location of the nucleus was confirmed by Hoechst staining. Immunofluorescent staining intensity with anti-p120 antibody was measured in a defined region (diameter 1.5 μm) in the nucleus or cytosol, and the ratio of nucleus/cytoplasm staining intensity was analyzed in 15 cells per transfection. *, significantly higher than control cells (p values < 0.01).*

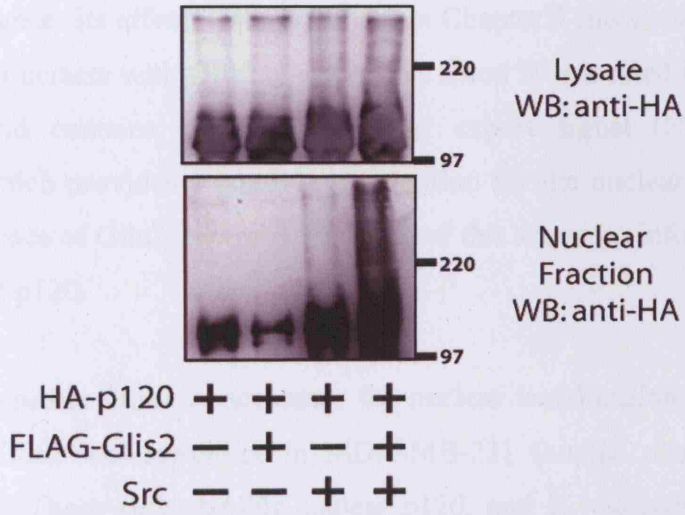

Figure 4.3 Examination of the localisation of p120 by nuclear fractionation. *FLAG-Glis2, HA-p120 and Src were expressed in HEK293 cells, and total cell lysates and nuclear fractions were examined by Western blotting using anti-HA antibody. This blot is representative of two separate experiments.*

To confirm the immunofluorescence data, nuclear fractionation assays were performed in HEK293 cells expressing FLAG-Glis2, HA-p120 and Src. Nuclear and cytoplasmic fractions were used for SDS-PAGE and analysed by Western blotting using anti-HA antibody. In the presence of Src, p120 in the nuclear fraction was slightly increased. The enrichment of p120 in the nuclear fraction was further increased in cells expressing both Glis2 and Src, consistent with the immunofluorescence data (Figure 4.3). What was inconsistent, however, was the slight decrease in the nuclear staining of p120 by nuclear fractionation, in the presence of Glis2 alone (Figure 4.3). By immunofluorescence, this combination led to a redistribution of p120 into the nucleus of a small proportion of the transfected cells, though not as convincingly as in the presence of Src as well (Figures 4.1 and 4.2). The reason for this discrepancy is not clear. It could be that nucleocytoplasmic shuttling of p120 is governed by other factors that were altered in this experimental setting, that were unaccounted for. Alternatively, the harvest of nuclei in this experiment could have been affected, resulting in the loss of nuclear protein. Further repetitions of this experiment would help to address this point.

Taken together, these data indicate that Glis2 promotes nuclear translocation of p120 and that Src enhances its effect. Data presented in Chapter 3 shows that the C-terminal portion of p120 interacts with Glis2 (Figure 3.6). Exon B is located in the C-terminal tail of p120 and contains a putative nuclear export signal (NES, Figure 1.4, Introduction), which provides a possible explanation for the nuclear accumulation of p120 in the presence of Glis2, whereby shielding of this sequence information prevents nuclear export of p120.

To investigate whether Glis2 is necessary for nuclear translocation of p120, siRNA knockdown of Glis2 was attempted in MDA-MB-231 (human mammary epithelial carcinoma) cells. These cells exhibit nuclear p120, and it was hypothesized that if Glis2 expression is required for nuclear translocation of p120, then in siRNA transfected cells, p120 would be excluded from the nucleus. siRNA oligos directed against human Glis2 were obtained from Qiagen and transfected into MDA-MB-231 cells. Blotting with anti-Glis2 antibody showed that knockdown was not successful in this cell line (Figure 4.4). A non-silencing oligo was used as a negative control, and as a positive control, cells were treated with an siRNA oligo directed against MAPK.

Blotting with anti-MAPK antibody showed the siRNA transfection was efficient as MAPK levels were successfully knocked down and anti-tubulin staining showed that equal protein levels were present in each sample (Figure 4.4). The Glis2 siRNA oligos were also transfected in HEK293 cells, but knockdown was not seen in this cell line either (data not shown).

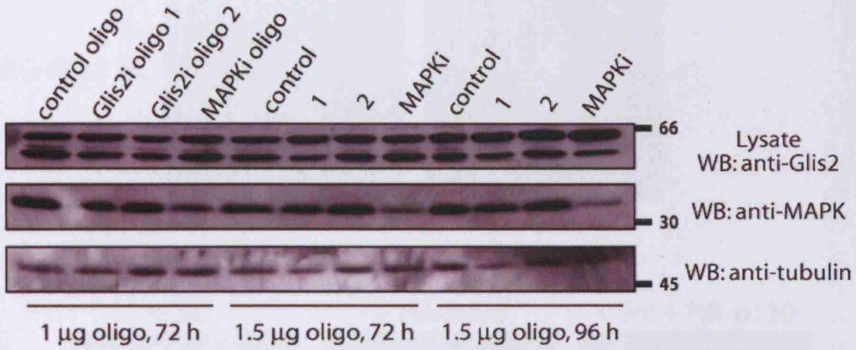

Figure 4.4 Attempted knockdown of Glis2 using siRNA oligos. *MDA-MB-231 cells were transiently transfected with a control non-silencing RNAi oligo, two RNAi oligos for Glis2, and a positive control RNAi oligo for MAPK. Oligos were transfected using 1 µg or 1.5 µg and were incubated for 72 h or 96 h, as indicated. Lysates were examined by Western blotting using anti-Glis2, anti-MAPK and anti-tubulin antibodies. These blots are representative of two separate experiments.*

4.2.2 Both Glis2 and Glis2 ΔC localize to the nucleus

FLAG-Glis2 and FLAG-Glis2 ΔC were each expressed in HEK293 cells and their localisations were examined by immunofluorescence. FLAG-Glis2 primarily localizes to the nucleus, but also with some diffuse cytoplasmic staining (Figure 4.5A). FLAG-Glis2 ΔC shows a much clearer localisation to the nucleus only (Figure 4.5A), indicating that it may not localize in the cytoplasm. To investigate whether the localisation of Glis2 is affected by the expression of p120 and Src, HEK293 cells co-expressing FLAG-Glis2, HA-p120 and Src were analysed by immunofluorescence. Co-expression of HA-p120 with FLAG-Glis2 and staining for Glis2 shows that its nuclear localisation is maintained (Figure 4.5B). The same result is seen when both HA-p120 and Src are co-expressed with FLAG-Glis2, indicating that the interaction with p120 does not alter the distribution of Glis2 (Figure 4.5B).

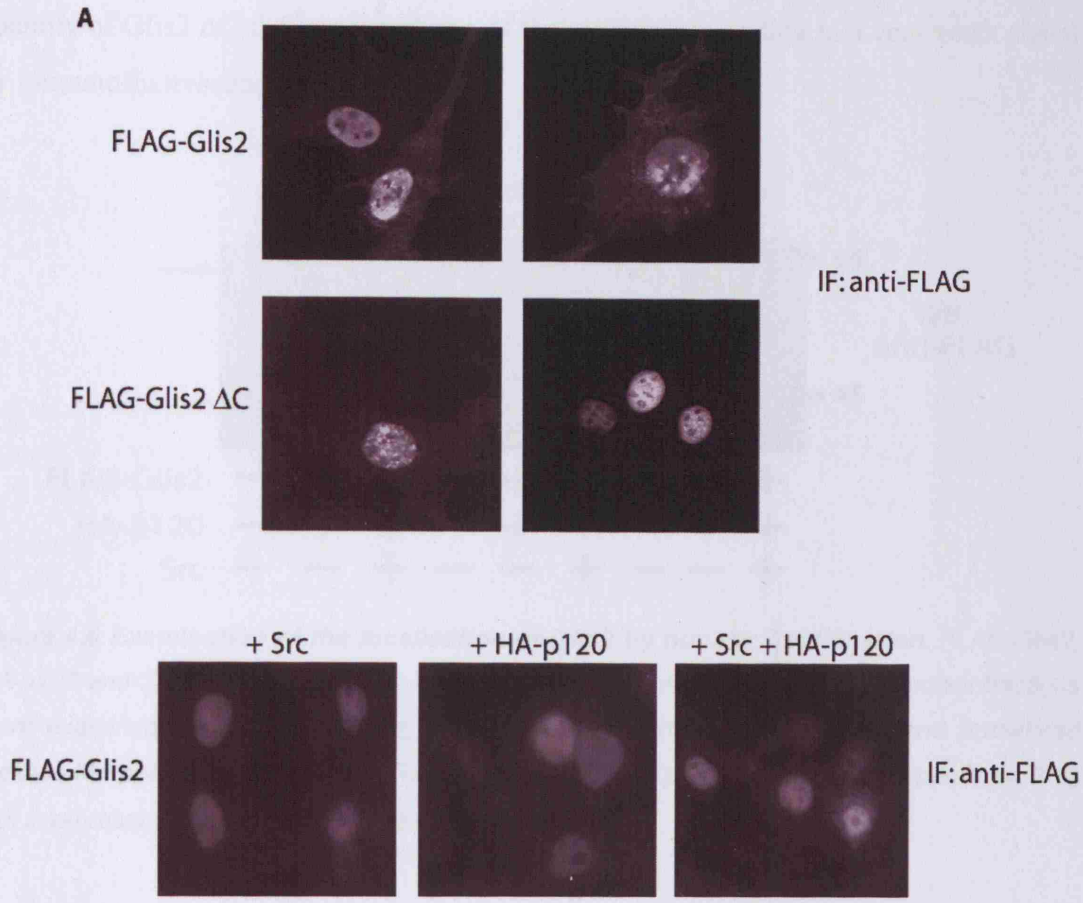

Figure 4.5 Immunofluorescent staining of exogenous Glis2. A *FLAG-Glis2 or FLAG-Glis2 ΔC were transiently transfected in HEK293 cells.* ***B*** *FLAG-Glis2 was transiently transfected into HEK293 cells with Src, HA-p120 or both. The localization of Glis2 was examined by epifluorescent microscopy using anti-FLAG antibody.*

Assessing the localisation of Glis2 biochemically was also undertaken by the nuclear fractionation of HEK293 cells expressing FLAG-Glis2, HA-p120 and Src. The localisation of marker nuclear and cytoplasmic proteins was examined to confirm the efficiency of the fractionation technique (Section 2.3.7, Materials and Methods). Nuclear and cytoplasmic fractions were used for SDS-PAGE and analysed by Western blotting using anti-FLAG antibody. Using this technique, it seems that in fact both Glis2 and Glis2 ΔC are found in the cytoplasm as well as in the nucleus, even in the presence of p120 and Src (Figure 4.6). This suggests that the cleaved form of Glis2 can actually shuttle between the nucleus and the cytoplasm. This difference between the immunofluorescence result and the nuclear fractionation could be due to the higher concentration of Glis2 ΔC in the smaller area of the nucleus compared to a smaller

quantity of Glis2 ΔC the larger volume of the cytoplasm, leading to a very weak signal by immunofluorescence.

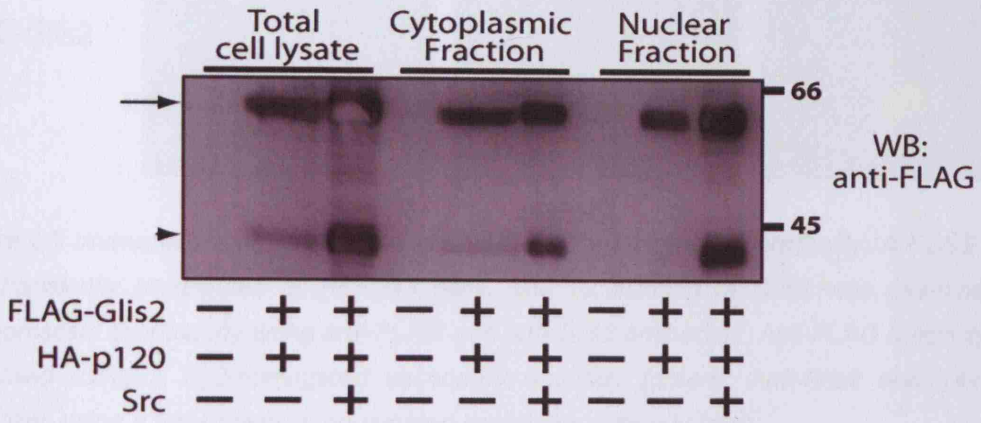

Figure 4.6 Examination of the localisation of Glis2 by nuclear fractionation. *FLAG-Glis2, HA-p120 and Src were expressed in HEK293 cells, and total cell lysates and nuclear fractions were examined by Western blotting using anti-FLAG antibody. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

To study the localisation of endogenous Glis2, anti-Glis2 antibody (that is known to be useful for Western blotting, described in Section 3.2.3) was tested to see if it could be used in immunofluorescence studies. HEK293 cells were transfected with FLAG-Glis2 and stained with anti-FLAG antibody and anti-Glis2 antibody. FLAG staining was identified with a Cy2-conjugated secondary antibody and Glis2 staining with a Rhodamine X conjugated antibody. The anti-Glis2 antibody was able to detect overexpressed Glis2 as shown by co-localisation of the green (Cy2) and red (Rhodamine X) staining (Figure 4.7). However, untransfected cells in the same field of view showed very little red staining, indicating that the anti-Glis2 antibody is not sufficiently sensitive to pick up endogenous levels of Glis2 (Figure 4.7). It was therefore concluded that the anti-Glis2 antiserum was not functional for immunofluorescence studies. Future experiments could be conducted using the TSA (Tyramide Signal Amplification) reagent for a more sensitive readout.

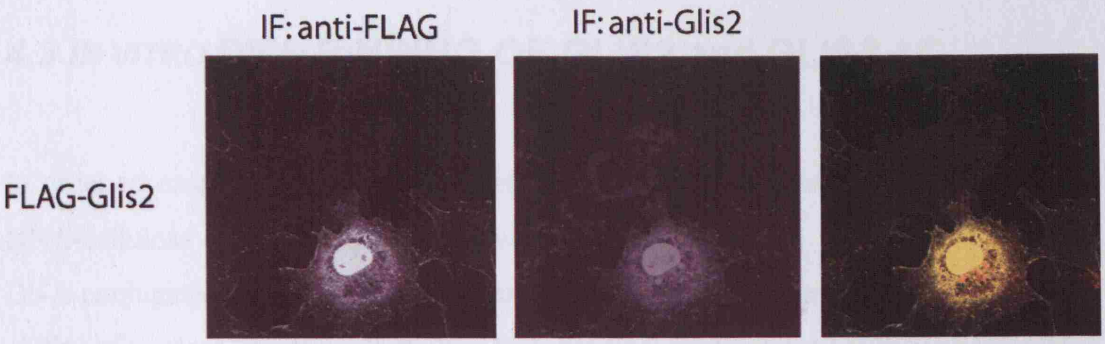

Figure 4.7 Immunofluorescent staining of Glis2 using anti-Glis2 antibody. A *FLAG-Glis2 was transiently transfected in HEK293 cells. The localization of Glis2 was examined by epifluorescent microscopy using anti-FLAG and anti-Glis2 antibodies. Anti-FLAG reactivity was visualised using a Cy2-conjugated secondary antibody (green). Anti-Glis2 reactivity was visualised using a Rhodamine X-conjugated secondary antibody (red).*

4.3 *IN VITRO* DNA BINDING OF GLIS2 and GLIS2 ΔC

In order to examine whether both Glis2 and Glis2 ΔC are competent to bind DNA, DNA-cellulose binding experiments were carried out, using sonicated calf thymus DNA conjugated to cellulose. This assay is widely used to examine the DNA-binding ability of nuclear proteins when the DNA binding sites are not identified (Johnson et al., 2004; Ritt et al., 1998; Surmacz et al., 2002). This was useful here, since it is possible that the cleaved form of Glis2 has a different DNA-binding specificity to the full-length protein, which is known to bind to the GLI Response Element (GLI RE) (Lamar et al., 2001; Zhang et al., 2002). Indeed, both the full-length and cleaved forms of Glis2 may bind to other sites on DNA that have not yet been characterized.

4.3.1 Both Glis2 and Glis2 ΔC bind to DNA *in vitro*

To assess whether FLAG-Glis2 and FLAG-Glis2 ΔC can bind to DNA-cellulose, these constructs were expressed in HEK293 cells with HA-p120 and Src. Cell lysates and precipitates from the DNA-binding assay were used for SDS-PAGE and analysed by Western blotting using anti-FLAG antibody. Both Glis2 and Glis2 ΔC were competent to bind DNA-cellulose in this experiment and did not bind to cellulose alone (Figure 4.8A). Quantification of band intensities shows that co-expression of p120 and Src does not affect the affinity of either Glis2 or Glis2 ΔC for DNA (Figure 4.8B). This is consistent with the fact that the p120 binding region of Glis2 is outside the zinc finger domains (Figure 3.1). These data suggest that Glis2 can potentially bind to both p120 and DNA at the same time, in contrast to the p120 Kaiso interaction (Kelly et al., 2004; Kim et al., 2004; Park et al., 2005).

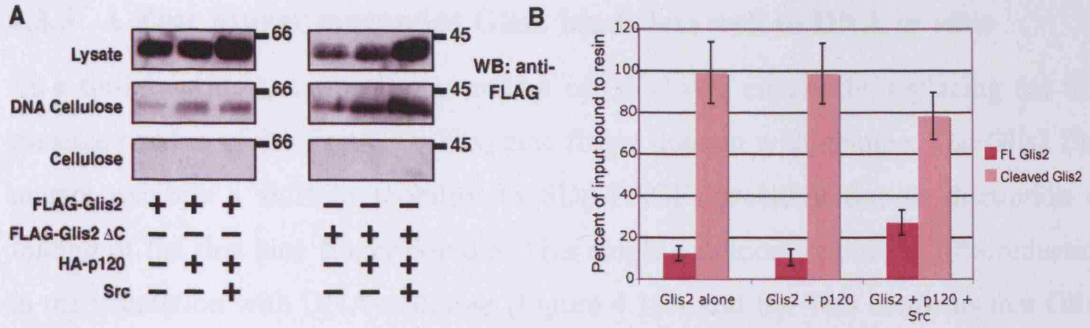

Figure 4.8* In vitro *DNA binding of Glis2 and Glis2 ΔC. A *Lysates of HEK293 cells expressing either FLAG-Glis2 or FLAG-Glis2 ΔC with HA-p120 and Src were incubated with DNA-cellulose or cellulose beads, followed by extensive washing. The amount of bound Glis2 was examined by Western blotting using anti-FLAG antibody. These blots are representative of three separate experiments.* ***B*** *Quantification of DNA binding abilities of Glis2 and Glis2 ΔC. Band intensities were determined by densitometry. Differences between percent of input protein (dark pink, Glis2; light pink, Glis2 ΔC) bound to DNA in the presence or absence of p120 and Src were not statistically significant.*

4.3.2 DNA but not RNA inhibits the binding of Glis2 and Glis2 ΔC to DNA-cellulose

To confirm the specificity of the interaction with DNA, cell lysates were preincubated with increasing amounts of sonicated calf thymus DNA or calf liver RNA. As expected, RNA was unable to affect binding of Glis2 or Glis2 ΔC with DNA-cellulose, whereas preincubation with DNA successfully inhibited the binding of Glis2 and Glis2 ΔC to the DNA-cellulose (Figure 4.9). This indicates a specific interaction between DNA and Glis2 or Glis2 ΔC, rather than a non-specific ionic interaction.

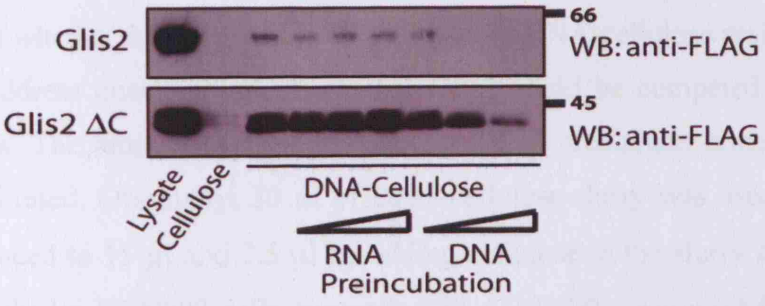

Figure 4.9 DNA, but not RNA, inhibits the interaction of Glis2 and Glis2 ΔC with DNA-cellulose beads. *Lysates of HEK293 cells expressing FLAG-Glis2 or FLAG-Glis2 ΔC were pre-incubated for 30 min with 1, 5, or 20 μg of DNA or RNA, followed by incubation with DNA-cellulose beads. The amount of bound Glis2 was examined by Western blotting using anti-FLAG antibody. These blots are representative of three separate experiments.*

4.3.3 A Zinc Finger mutant of Glis2 binds less well to DNA *in vitro*

As a further control, a zinc finger mutant of Glis2 was created by replacing the first cysteine residue of the first Cys_2-His_2 zinc finger domain with alanine. The Glis2 ZnF mutant exhibits a shift in mobility in SDS-PAGE, probably due to disruption of folding of the first zinc finger domain. This single mutation induced a 50% reduction in the interaction with DNA-cellulose (Figure 4.10A and B). This confirms that Glis2 binds to DNA specifically through its zinc finger domains.

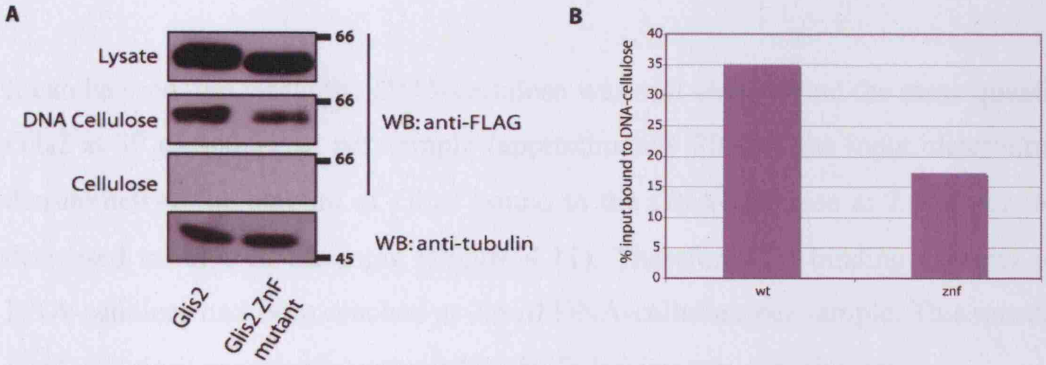

Figure 4.10 A zinc finger mutant of Glis2 shows a weaker interaction with DNA. ***A*** *Lysates of HEK293 cells expressing FLAG-Glis2 or FLAG-Glis2 ZnF mutant were incubated with DNA-cellulose or cellulose beads, and the amount of bound Glis2 was examined by Western blotting using anti-FLAG antibody. Equal protein loading was confirmed by blotting with anti-tubulin antibody.* ***B*** *Band intensities were analysed using densitometry and the percentage of the input bound to DNA-cellulose was expressed as a percentage.*

4.3.4 Glis2 is competed off DNA-cellulose by GLI1

I investigated whether it was possible to saturate the DNA-cellulose resin with Glis2, in order to address questions about whether Glis2 could be competed off DNA by other proteins. The amount of DNA-cellulose used to precipitate proteins from cell lysates was titrated. Originally, 30 µl of DNA-cellulose slurry was used per sample. This was reduced to 15 µl and 7.5 µl by adding cellulose to the slurry at 1:1 and 3:1 ratios, respectively. HEK293 cells expressing FLAG-Glis2 were used for the DNA-cellulose binding assay as normal with these three amounts of DNA-cellulose slurry (Figure 4.11).

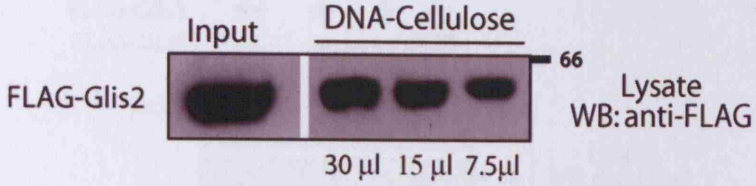

Figure 4.11 Titration of the amount of DNA-cellulose used per sample. *FLAG-Glis2 was expressed in HEK293 cells, and lysates were incubated with 30, 15, or 7.5 µl of DNA-cellulose beads, followed by extensive washing. The amount of bound Glis2 was examined by Western blotting using anti-FLAG antibody. Band intensities were analysed by densitometry. This blot is representative of two separate experiments.*

It can be seen that while the DNA-cellulose was still able to bind the same quantity of Glis2 at 30 µl and 15 µl per sample (approximately 70% of the input, determined by densitometry), the amount of Glis2 bound to the DNA-cellulose at 7.5 µl per sample decreased to 40% of the input (Figure 4.11). Therefore, the binding capacity of the DNA-cellulose had been reached at 7.5 µl DNA-cellulose per sample. This quantity of DNA-cellulose per sample was used in the following two experiments.

It has been reported that Glis2 can recognise the GLI Response Element (GLI RE) in *in vitro* reporter assays (Zhang et al., 2002). It was therefore hypothesized that GLI and Glis2 would be in competition for the GLI RE. To examine this using the DNA-binding assay, FLAG-Glis2 was expressed in HEK293 cells with increasing amounts of GLI1. As expected, GLI1 is able to compete Glis2 off DNA-cellulose (Figure 4.12). However, some Glis2 remains bound to the DNA-cellulose even at high expression levels of GLI1, thus leaving open the possibility that Glis2 has alternative binding sites on DNA. As shown in the previous chapter, in Figure 3.7, Glis2 does not bind to GLI1, therefore an indirect association with DNA via GLI1 is not thought to be the explanation for the residual Glis2 binding to the DNA-cellulose.

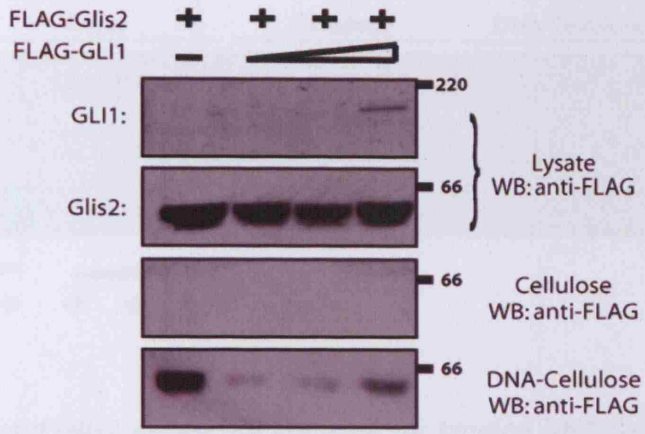

Figure 4.12 Effect of GLI1 overexpression of DNA-binding ability of Glis2. *FLAG-Glis2 was expressed in HEK293 cells with increasing amounts of FLAG-GLI1. Lysates were incubated with DNA-cellulose or cellulose beads, followed by extensive washing. The amount of bound Glis2 was examined by Western blotting using anti-FLAG antibody. 1, 5, and 10 μg of pcDNA-FLAG-GLI1 were used for transfection. The FLAG-GLI1 expresses at a low level in HEK293 cells, so reactivity by Western blotting is not detected easily. The effect on the DNA-binding ability of Glis2, however, indicates that the GLI1 is expressed, despite not appearing clearly on the blot. These blots are representative of two separate experiments.*

4.3.5 Glis2 and Glis2 ΔC do not compete for binding DNA *in vitro*

To investigate whether Glis2 and Glis2 ΔC compete for binding of the same DNA sequences, FLAG-Glis2 ΔC was expressed in HEK293 cells with increasing amounts of FLAG-Glis2. The amount of Glis2 ΔC bound to the DNA-cellulose did not decrease in the presence of high levels of Glis2 (Figure 4.13). Thus it appears that there is no competition for binding DNA between Glis2 and Glis2 ΔC, suggesting that the cleaved form of Glis2 may have alternative binding sites. This may be due to a lower specificity of interaction between Glis2 ΔC and DNA, that may result from containing four, rather than five, zinc finger domains.

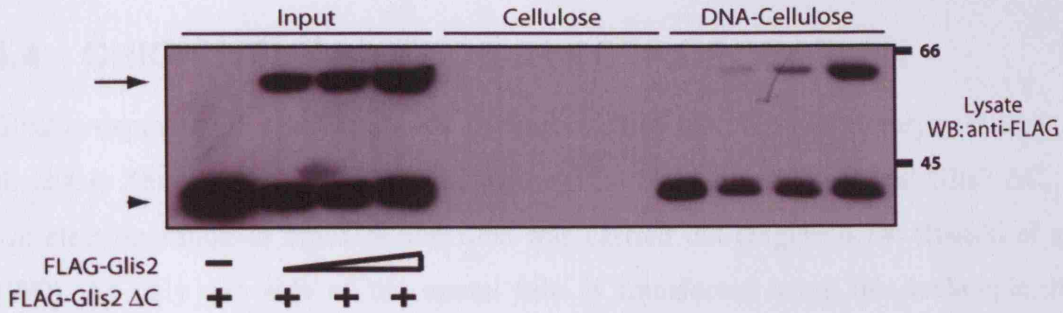

Figure 4.13 Glis2 and Glis2 ΔC do not compete for binding DNA-Cellulose. *FLAG-Glis2 ΔC was expressed in HEK293 cells with an increasing amount of FLAG-Glis2. Lysates were incubated with DNA-cellulose or cellulose beads, followed by extensive washing. The amount of bound Glis2 and Glis2 ΔC was examined by Western blotting using anti-FLAG antibody. 2.5, 5, and 10 μg of pcDNA-FLAG-Glis2 were used for transfection. The arrow and arrowhead indicate the positions of full-length Glis2 and its cleavage product Glis2 ΔC, respectively. This blot is representative of two separate experiments.*

4.4 CHICK NEURAL TUBE ELECTROPORATION

Glis2 is expressed in the neural tube in mouse, chick and *Xenopus* embryos (Lamar et al., 2001; Zhang et al., 2002). To investigate the function of Glis2 and Glis2 ΔC, *in ovo* electroporation of chick neural tube was carried out (Figure 4.14; (Itasaki et al., 1999). As only one side of the neural tube is transfected using this technique, the untransfected side can be used as a wild type control.

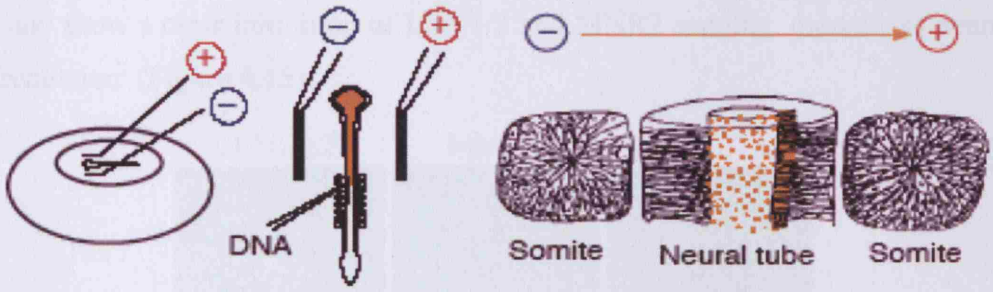

Figure 4.14 Chick* in ovo *electroporation. *A window is opened in the eggshell, and a DNA solution is injected into the neural tube. An electrode is placed either side of the neural tube of the embryo, and the DNA is transferred unilaterally to the side of the positive pole by application of electric pulses. Embryos are cultured* in ovo *and assayed for gene expression at later stages. Diagram from Itasaki et al., Nature Cell Biol, 1999.*

A previous report showed that expression of *Xenopus* Glis2 in chick neural tube affected neuronal differentiation (Lamar et al., 2001). Therefore, the effects of Glis2, p120 and Glis2 ΔC on neuronal differentiation were tested. In the experiments reported here, mouse cDNAs of Glis2, Glis2 ΔC and p120 were used.

Glis2 is structurally related to the GLI family of transcription factors, which are the main transducers of Sonic Hedgehog signalling. Overexpression of GLI proteins is known to affect dorso-ventral patterning of neuronal subtypes in the spinal cord in developing embryos (Briscoe and Ericson, 2001; Stamataki et al., 2005). As Glis2 has been shown to interact *in vitro* with the GLI binding site on DNA (Lamar et al., 2001), the effect of transfection of Glis2 and Glis2 ΔC on markers of dorso-ventral patterning in the neural tube was also examined.

4.4.1 Initial test of Glis2 expression in chick neural tube

A collaboration with James Briscoe at the National Institute for Medical Research was established. An initial test of Glis2 expression in the neural tube of chick embryos was carried out (Figure 4.15, by kind permission of J. Briscoe). By electroporating Glis2 with a vector containing GFP, transfected cells could be identified. Embryos were stained for a number of markers of dorso-ventral patterning and for markers of neuronal differentiation. This initial experiment showed that cells overexpressing Glis2 may have a mild effect on a marker of Shh signalling (transcription factor Nkx 2.2), and show a clear inhibition of Lim 1/2 and MNR2 staining, markers of neuronal differentiation (Figure 4.15).

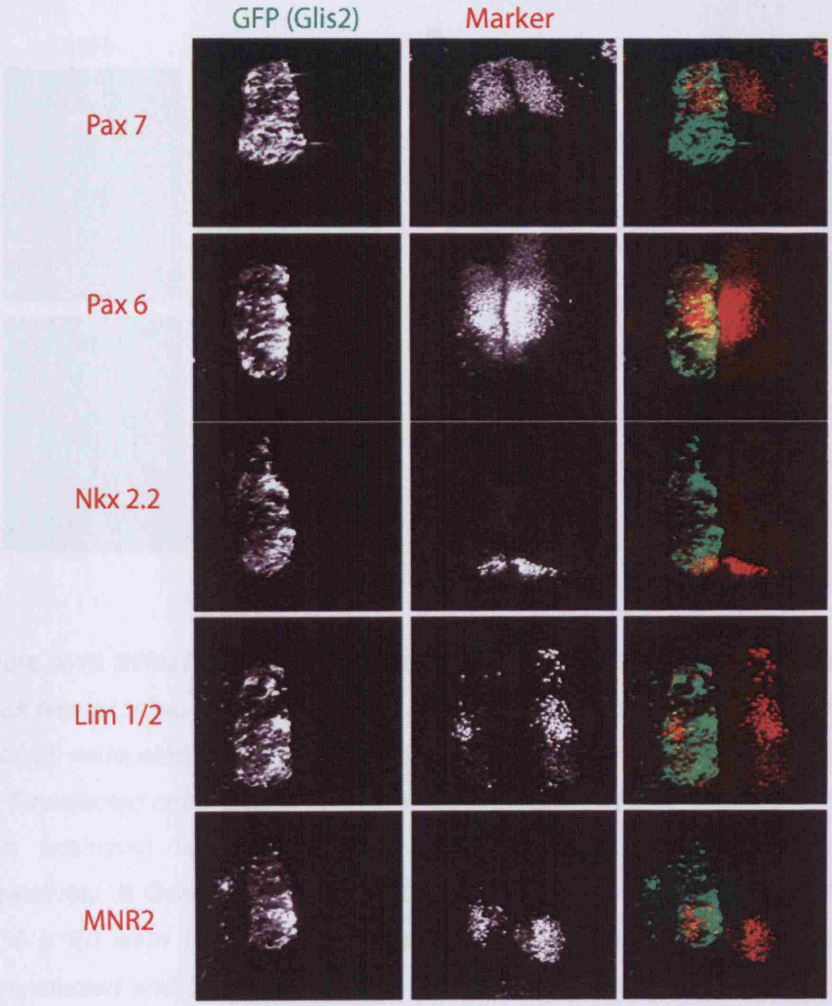

Figure 4.15 Chick* in ovo *electroporation of Glis2. *Staining of various markers of Shh signalling and neuronal differentiation. HH stage 10-12 embryos were electroporated with Glis2, and incubated until HH stage 22. Transfected cells were identified by co-expression of GFP. Expression of GFP, Pax 7, Pax 6, Nkx 2.2, Lim 1/2, and MNR2 were examined by confocal microscopy using the relevant antibodies. By kind permission of J. Briscoe.*

These data provided the basis for a more detailed investigation of the role of Glis2 in Shh signalling and neuronal differentiation.

4.4.2 Effect of Glis2 on Lim 1/2 expression pattern

Lim 1/2 are transcription factors which are expressed in post-mitotic neurons in the developing neural tube (Eisen, 1999). Staining for Lim 1/2 therefore gives an indication of which cells in the developing neural tube have differentiated into their neuronal subtype. Chick neural tubes were electroporated with either Glis2, Glis2 ΔC, p120, Glis2 & p120, or Glis2 ΔC & p120. Sections were stained for GFP and Lim 1/2.

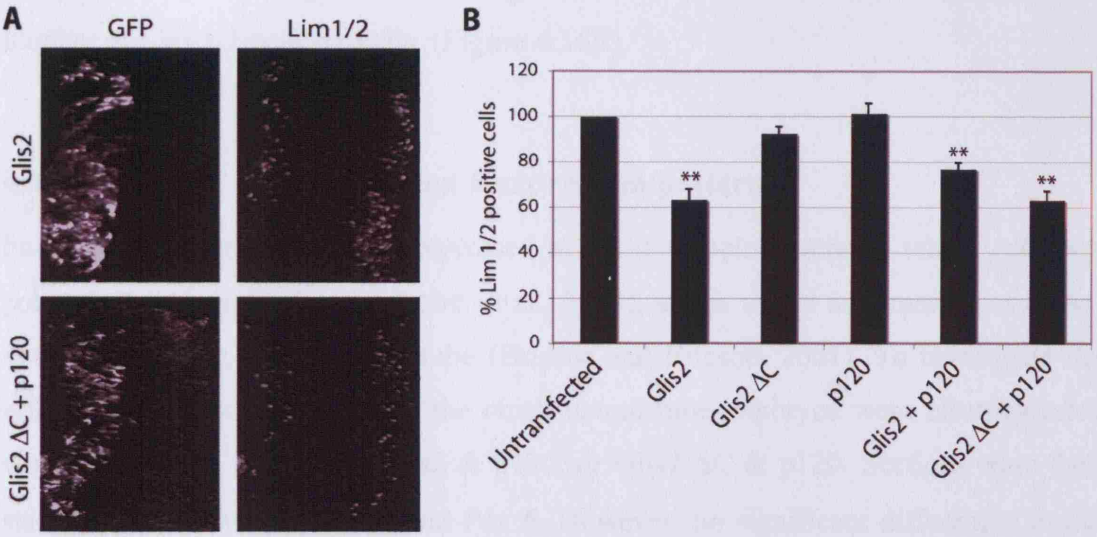

Figure 4.16 Effect of expression of Glis2 and Glis2 ΔC on neuronal differentiation in chick neural tube. A *Staining of Lim 1/2, a marker of neuronal differentiation. HH stage 10-12 embryos were electroporated with Glis2, or Glis2 ΔC and p120, and incubated until HH stage 22. Transfected cells were identified by co-expression of GFP. Expression of GFP and Lim 1/2 were examined by confocal microscopy using anti-GFP and anti-Lim 1/2 antibodies, respectively.* ***B*** *Quantification of Lim 1/2 staining. Glis2, Glis2 ΔC, p120, Glis2 & p120, or Glis2 ΔC & p120 were transfected in neural tubes. The number of cells positive for Lim 1/2 in untransfected and transfected neural tubes were counted from 20 sections of three separate experiments, with at least 6 embryos sectioned per experiment. Results are expressed as a percentage relative to untransfected cells. **, significantly lower than untransfected cells (p values < 0.001).*

In neural tubes expressing Glis2 alone, a decrease in the number of Lim 1/2 positive cells was observed (Figure 4.16A), indicating an inhibition of neuronal differentiation. This effect was also seen when Glis2 ΔC was co-expressed with p120, although expression of Glis2 ΔC alone had no significant effect (Figure 4.16A and B). This indicates that the cleaved form of Glis2 is still capable of affecting transcriptional events despite the loss of one zinc finger domain, but requires p120 for this function. When p120 was co-expressed with full-length Glis2, there was no significant difference in the number of Lim 1/2 positive cells compared with neural tubes expressing Glis2 alone. The decrease in the number of Lim 1/2 positive cells in Glis2-, Glis2/p120- and Glis2 ΔC/p120-expressing neurons was statistically significant compared to the wild type control (Figure 4.16B). p120 alone had no effect on the number of Lim 1/2 positive cells (Figure 4.16B).

4.4.3 No effect of Glis2 on Pax 6 expression pattern

Pax 6 is a transcription factor expressed in the developing embryo, which acts as a potent cell fate determinant (Haubst et al., 2004), and is useful as a marker of dorso-ventral patterning in the neural tube (Briscoe and Ericson, 2001). To investigate the effect of Glis2 on patterning of the chick neural tube, embryos were electroporated with Glis2, Glis2 ΔC, p120, Glis2 & p120, or Glis2 ΔC & p120. Sections were then stained for expression of GFP and Pax 6. However, no significant differences in the expression pattern of Pax 6 were observed (Figure 4.17).

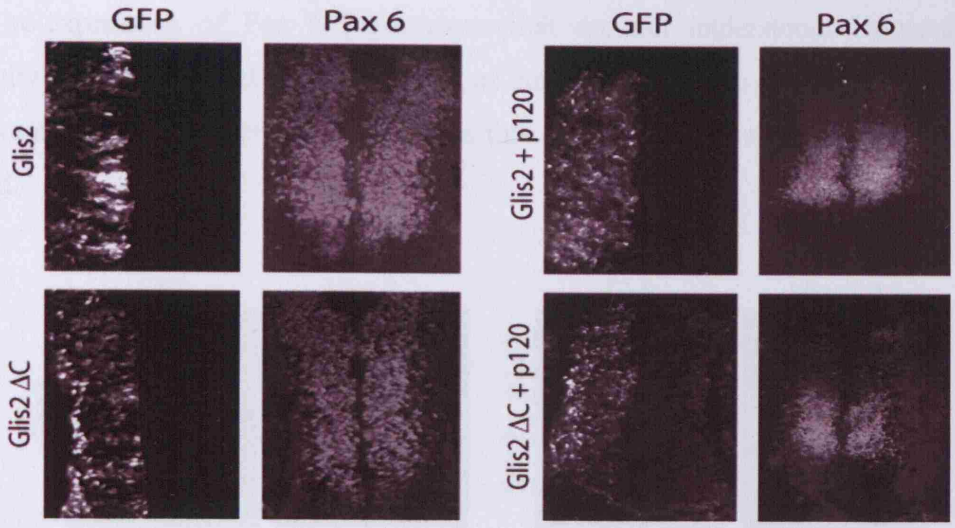

Figure 4.17 Effect of expression of Glis2 and Glis2 ΔC on patterning of Pax 6 in chick neural tube. *Staining of Pax 6, a marker of neuronal patterning. HH stage 10-12 embryos were electroporated with Glis2, Glis2 ΔC, Glis2 & p120, or Glis2 ΔC and p120, and incubated until HH stage 22. Transfected cells were identified by co-expression of GFP. Expression of GFP and Pax 6 were examined by confocal microscopy using anti-GFP and anti-Pax 6 antibodies, respectively. These images are representative of three separate experiments, with at least 6 embryos sectioned per experiment.*

4.4.4 Possible effect of Glis2 on Nkx 2.2 expression pattern

Nkx 2.2 is a transcription factor that shares a progenitor domain boundary with Pax 6 in the developing neural tube (Briscoe and Ericson, 2001). In other words, Pax 6 and Nkx 2.2 are cross-repressive and so alteration of the expression pattern of one will affect the patterning of the other. Since no significant effect was seen on Pax 6 expression upon overexpression of Glis2, it was expected that there would not be a difference in Nkx 2.2 expression either. However, as shown in Figure 4.18, some repression of Nkx 2.2 staining was observed following both Glis2 and Glis2 ΔC overexpression. This data is difficult to interpret, since there was no concomitant change in the Pax 6 staining as described above. It is possible that this result indicates a minor effect that is not strong enough to affect the cross-repression of Pax 6. Alternatively, it could be a consequence of the timing – it is possible the electroporations (where there is ventral expression) were done in neural tubes that were beyond the point where Pax 6 is sensitive to re-expression (J. Briscoe, personal

communication). Another possibility could be that the expression of Glis2 does not allow re-expression of Pax 6 for reasons that are not understood. However, the difficulty in obtaining sufficient numbers of embryos that were electroporated in this most ventral part of the neural tube means that analysis of these results is necessarily limited.

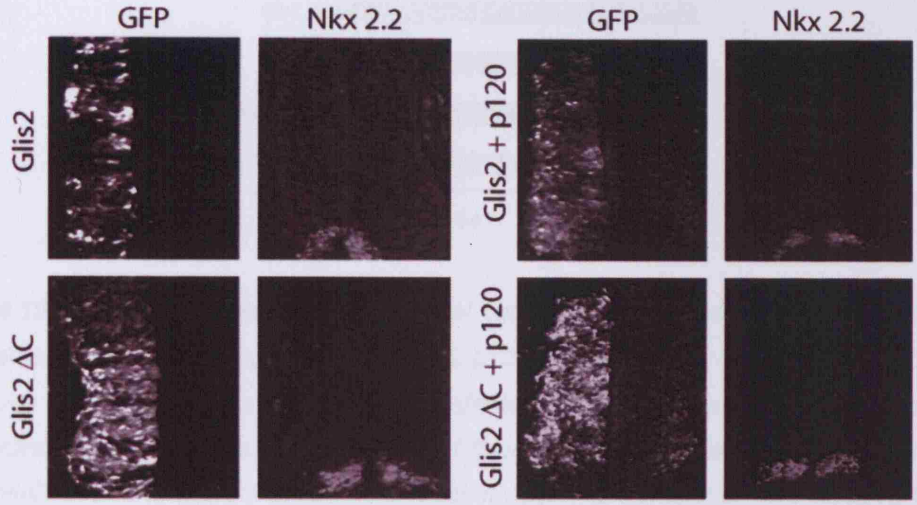

Figure 4.18 Effect of expression of Glis2 and Glis2 ΔC on patterning of Nkx 2.2 in chick neural tube. *Staining of Nkx 2.2, a marker of neuronal patterning. HH stage 10-12 embryos were electroporated with Glis2, Glis2 ΔC, Glis2 & p120, or Glis2 ΔC and p120, and incubated until HH stage 22. Transfected cells were identified by co-expression of GFP. Expression of GFP and Nkx 2.2 were examined by confocal microscopy using anti-GFP and anti-Nkx 2.2 antibodies, respectively. These images are representative of three separate experiments, with at least 6 embryos sectioned per experiment.*

4.4.5 No effect of Glis2 on BrdU incorporation

It was observed that some embryos transfected with Glis2 appeared to have a smaller neural tube on the Glis2-expressing side (Figure 4.15). To address whether the cause of this smaller size was an inhibition of proliferation in cells expressing Glis2, BrdU incorporation of these cells was examined. BrdU is incorporated into newly synthesized DNA during S phase of mitotic cells. Embryos were given a 1 hr pulse of BrdU to the heart before sacrificing. Sections were stained for GFP and BrdU. Counting BrdU positive cells on transfected and untransfected sides of the neural tube showed that there was no statistical difference in numbers of mitotic cells (Figure 4.19). This indicates that Glis2 does not inhibit proliferation, and any slight difference

in size of neural tubes is due to some other factor – for example an increase in apoptosis. However, attempts to quantify apoptosis in the neural tube with TUNEL staining of transfected embryos were unsuccessful.

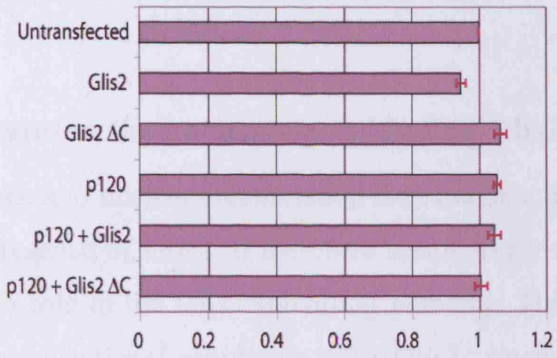

Figure 4.19 BrdU treatment of chick neural tubes. *Quantification of BrdU incorporation. Chick neural tubes expressing Glis2, Glis2 ΔC, p120, Glis2 & p120, or Glis2 ΔC & p120 were pulsed with BrdU for 1 h before sacrifice. Transfected cells were identified by co-expression of GFP. Expression of GFP and incorporation of BrdU were examined by confocal microscopy using anti-GFP and anti-BrdU antibodies, respectively. The number of cells positive for BrdU incorporation in transfected and untransfected neural tubes were counted from 20 sections of two separate experiments, with at least 6 embryos sectioned per experiment. Results are expressed as a proportion relative to untransfected cells. None of the data were significantly different from the control untransfected cells.*

4.5 DISCUSSION

This chapter describes an investigation of the role of p120 and Glis2 together in the nucleus. The implications of these results are discussed below.

4.5.1 p120 translocates to the nucleus upon binding Glis2

The immunofluorescence and nuclear fractionation data indicate that p120 translocates to the nucleus in the presence of Glis2. It therefore seems likely that p120 is required in the nucleus to play a role in the Glis2 signalling pathway. This suggests that p120 may be involved in transcriptional regulation with Glis2. Analyses of the ability of p120 to interact with DNA in the presence of Glis2 using the DNA-cellulose binding assay were unsuccessful. A better approach would be to identify all DNA binding sites of Glis2 and perform gel shift and super-shift assays in the presence or absence of p120.

It has been reported that NLS or NES sequences of p120 regulate its nucleocytoplasmic shuttling (Kelly et al., 2004; Roczniak-Ferguson and Reynolds, 2003; van Hengel et al., 1999). The evidence presented here indicates that co-expression of Glis2 and Src comprise a further mechanism for recruiting p120 into the nucleus. To assess the importance of Glis2 in this translocation of p120, knocking down expression of Glis2 using RNAi was tried. Despite several attempts, it was not possible to efficiently reduce Glis2 proteins levels. Thus whether Glis2 is essential to recruit p120 into the nucleus remains to be clarified.

The immunofluorescence data in Figure 4.1 shows a very clear enhancement of Glis2-induced nuclear localisation of p120 by the overexpression of Src. Thus Src fulfils at least two roles in the context of the p120/Glis2 interaction, leading to enhanced proteolytic processing of Glis2 (Chapter 3) and enhanced nuclear localisation of p120. In addition, the stabilisation of the Glis2 protein in the presence of p120 and Src may also be a consequence of Src phosphorylation of p120, Glis2 or some other component of the pathway. The mechanism by which these processes occur is not yet known. Future experiments that utilise kinase dead or temperature sensitive Src constructs may go some way to addressing the contribution of Src activity to these processes. In

addition, a p120 mutant with every tyrosine residue mutated to alanine would be a useful tool to address the contribution of Src phosphorylation to the p120/Glis2 interaction, and subsequent cleavage and relocation to the nucleus[1].

The importance of Src in augmenting the nuclear translocation of p120 seen here provides an interesting new angle on Src's links with the development of cancer and progression to metastases. If Src phosphorylation of p120 could concomitantly promote the disassembly of adherens junctions and the accumulation of p120 in the nucleus, when Glis2 is also present, this could provide a direct mechanism to link downregulation of cell-cell adhesion, and upregulation of target genes (the identity of which are so far undetermined) that may promote proliferative/invasive behaviour. This is discussed further in Chapter 6.

4.5.2 The interaction with p120 does not inhibit the *in vitro* DNA binding ability of Glis2 or Glis2 ΔC.

Both Glis2 and Glis2 ΔC are able to bind to DNA *in vitro*. This indicates that the cleaved form of Glis2 may still function as a transcription factor *in vivo*, despite the loss of the last zinc finger domain. p120 does not seem to inhibit the interaction of Glis2 or Glis2 ΔC with DNA, in contrast to the p120/Kaiso interaction (Daniel et al., 2002; Spring et al., 2005). For the p120/Kaiso interaction, EMSA and ChIP assays were employed to show that Kaiso binding to the *matrilysin* promoter was reduced in the presence of overexpressed p120 (Daniel et al., 2002; Spring et al., 2005). Whether p120 can alter the DNA-binding specificity of Glis2, and/or act to increase the levels of Glis2 ΔC which itself has a different binding specificity, will be interesting to investigate. Identification of p120/Glis2 target genes will allow a more detailed examination of this question.

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[1] A p120 construct with 8 tyrosine-alanine mutations at positions known to be targeted by Src has been produced by our collaborator Margaret Frame. However, preliminary reports from her lab indicate that other tyrosine residues of p120 substitute when the preferred tyrosine residues are not available.

4.5.3 Glis2 affects neuronal differentiation in the chick neural tube

Data presented here indicate that Glis2 can affect differentiation pathways in neuronal cells. This supports recent reports suggesting that p120 has a role in development of the embryonic axis (Kim et al., 2004; Park et al., 2005). In these reports, it is shown that the p120/Kaiso interaction can cooperate with β-catenin/TCF signalling in the nucleus to regulate the activity of Wnt genes. The discovery that p120 has another binding partner involved in embryonic development points to the possibility that cadherin-associated regulation of gene expression is a multi-faceted signalling cascade involved in multiple pathways. Particularly interesting is the data that shows that Glis2 ΔC only has an effect on Lim 1/2 expression in the presence of p120. This is evidence that p120 is vital to the ability of this transcription factor to function. The fact that the same effect is seen as the full-length protein alone is intriguing. Intuitively, it would make sense for the cleaved form of the protein to have alternative targets and therefore different phenotypes. Clearly, further dissection of this signalling pathway is required to pinpoint the differences between the transcriptional regulation of Glis2 and Glis2 ΔC.

Lamar and colleagues report that overexpression of *Xenopus* Glis2 in chick neural tube can promote premature neuronal differentiation. By overexpressing Glis2, they report that progenitor cells withdraw from the cell cycle and differentiate as neurons. Most interestingly, in the context of the work presented in this thesis, in their report, no difference is seen in the level of Lim1/2 staining on the transfected side of the embryo compared to wildtype (Lamar et al., 2001). This is directly opposite to the results shown here. It is difficult to see how the apparent discrepancy between the phenotypes can be explained. One possible factor is that in their study the cDNA from *Xenopus* was used to electroporate chick embryos, whereas here the mouse cDNA was used. The *Xenopus* and mouse cDNAs only share 67% homology, with particular differences in the C-terminal tail of the protein (Lamar et al., 2001). Therefore it is possible that interaction partners of mouse Glis2 are not able to bind to the Xenopus homologue (or vice versa) and this difference in signalling partners may affect the outcome of overexpression of this protein in the neural tube. In order to fully understand the physiological role of Glis2 in neurogenesis, further genetic examination such as production of a knockout mouse will be required. This avoids the uncertainties that

arise when using cDNAs from a variety of species to address questions involving complex developmental signalling pathways.

4.5.4 Glis2 does not have a clear effect on dorso-ventral patterning in the chick neural tube

So far, the only known binding site of Glis2 on DNA is the GLI RE. This suggests that Glis2 may have some role in GLI signalling in the Sonic Hedgehog pathway. The results presented here, however, do not provide a clear picture of the ability of Glis2 to affect Shh signalling in the chick neural tube. It has been reported that aberrant expression of GLI proteins in the developing neural tube in chick leads to disruption of dorso-ventral patterning (Briscoe and Ericson, 2001; Stamataki et al., 2005). The data presented here indicates that there may be some minor effect on the patterning of the transcription factor Nkx 2.2, but that the cross-repression partner of Nkx 2.2, Pax 6, is unaffected. Therefore any effect that is exerted on this system by Glis2 is quite minor.

The most likely possibility for the mode of action of Glis2 is as a competitor for the GLI RE. By preventing GLI proteins from binding to target promoters, the downstream signalling would be dampened, leading to insufficient levels of transcription from these loci. Expression of proteins that rely on high levels of Shh signalling – i.e. those in the most ventral regions of the neural tube closest to the floor-plate releasing Shh, would be most affected (Figure 1.8, Introduction). This could explain why an effect is seen for Nkx 2.2 but not for Pax 6. Data from the Briscoe lab indicates there could indeed be some slight inhibitory effect by Glis2 on a GLI-reporter construct in luciferase assays (F. Ulloa, personal communication), supporting this hypothesis. Whether this is a mechanism employed *in vivo* has yet to be addressed.

CHAPTER 5

IDENTIFICATION OF NOVEL

HAKAI BINDING PROTEINS

5.1 INTRODUCTION

Hakai is an E3 ubiquitin ligase that has been shown to specifically target E-cadherin for internalisation and degradation (Fujita et al., 2002). There is good reason to believe that Hakai is involved in other pathways: firstly, it is ubiquitously expressed (Y. Fujita, unpublished data), whereas E-cadherin is mainly expressed in the epithelium; secondly, it has been shown to localize to the nucleus of MDCK and HEK293 cells (Y. Fujita, unpublished data), and thirdly, it is very common for E3-ligases to have several targets, for example, Mdm2 can ubiquitinate the β2-adrenergic receptor and p53, and recently it was shown Mdm2 can ubiquitinate E-cadherin itself (Haupt et al., 1997; Shenoy et al., 2001; Yang et al., 2005). A yeast 2-hybrid screen, using the proline-rich C-terminal portion of Hakai as bait, has yielded several potential binding partners for Hakai.

This chapter describes the cloning of these yeast 2-hybrid fragments into mammalian expression vectors, and verification of the specificity of binding of these proteins to Hakai. An investigation into the functional significance of these verified interactions was also carried out.

5.2 IDENTIFYING HAKAI INTERACTING PROTEINS

5.2.1 Yeast 2-hybrid using Hakai C-terminus as bait

Using the yeast 2-hybrid method, as described in Section 3.2.1, several potential interaction partners for Hakai were identified, using the C-terminus of Hakai as bait (amino acids 191-492; Figure 5.1) (Y. Fujita, unpublished data). cDNA libraries from human brain and mouse embryo were used. It was hoped that the high proline content of the C-terminus of Hakai indicated the existence of protein-protein interaction domains. The positive clones were sequenced and then analysed using the online BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the potential binding partners for Hakai (Table 5.1).

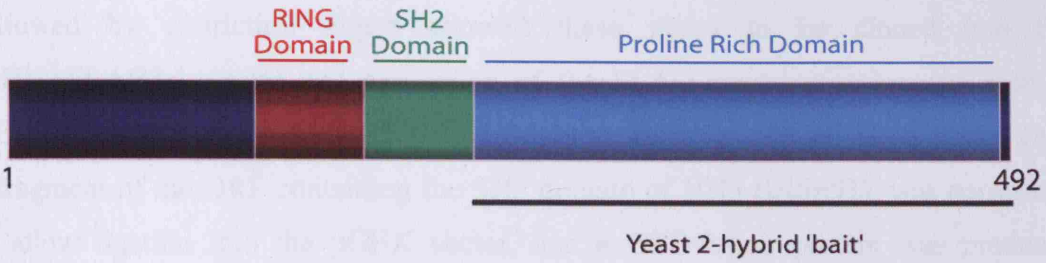

Figure 5.1 Domain structure of Hakai. *The RING finger domain contains the E3 ubiquitin ligase activity. The SH2 domain is responsible for binding to tyrosine phosphorylated E-cadherin. The C-terminal proline-rich domain was used as bait in a yeast 2-hybrid assay.*

Clone No.	Name of protein	Function
HH5	IRSp53	Insulin Receptor Substrate p53. Involved in cytoskeletal dynamics. (Yeh et al., 1996)
HH9	E1B-AP5	Adenovirus early 1B 55-kDa-associated protein. Implicated in nucleocytoplasmic mRNA transport. (Gabler et al., 1998)
HH31	Itch	E3 ubiquitin ligase. Involved in downregulation of E3 ubiquitin ligase Cbl. (Perry et al., 1998)
HH70	NADH dehydrogenase 4	Component of the mitochondrial electron transfer chain. (Chomyn et al., 1986)
HH82	AES	Amino-terminal enhancer of Split. Similar to Groucho, a drosophila transcription factor involved in neurogenesis. (Hou and Li, 1998)
HH97	Mena	Mouse homolog of Enabled. Involved in control of microfilament dynamics. (Gertler et al., 1996)
HH99	GZP1	GC-box-binding zinc finger protein. A Krüppel-like zinc finger protein. (Lisowsky et al., 1999)
HH101	KSRP	KH-type splicing regulatory protein. Involved in exon inclusion. (Min et al., 1997)

Table 5.1 *Details of clones that interacted with the C-terminus of Hakai in the yeast 2-hybrid screen.*

5.2.2 Making tagged constructs

Positive clones from the yeast 2-hybrid screen in the pACTII plasmid, (HH5, HH9, HH31, HH70, HH82, HH97, HH99, and HH101), were excised using restriction enzymes and ligated into the mammalian expression vector pcDNAFLAG3.1. Of these, only the cloning of HH101 resulted in a construct that gave detectable expression of the FLAG-tagged protein in HEK293 cells (Figure 5.2, **A**). PCR amplification of the open reading frame (ORF) of HH5 (IRSp53) and HH9 (E1B-AP5)

followed by restriction digest allowed these genes to be cloned into the pcDNAFLAG3.1 vector and expression of the FLAG-tagged proteins was seen in HEK293 cells (Figure 5.2, **A**). Restriction enzyme digest of pACTII-HH5, which gave a fragment of the ORF containing the SH3 domain of HH5 (IRSp53), was performed to allow ligation into the pGEX vector, and a GST-fusion protein was produced (Figure 5.2, **B**).

HH31 (Itch) and HH97 (Mena) were also cloned into the pGEX vector for production of GST-tagged proteins, but were poorly expressed using this vector system. pRK5-Myc-Itch and pBabe-AU-Mena were kindly received from D. Birnbaum and A. Hall, respectively. Expression of Myc-tagged Itch was obtained in HEK293 cells (Figure 5.2, **A**). However, expression of AU-tagged Mena was not observed in HEK293 cells, and attempts to amplify the ORF by PCR were also unsuccessful.

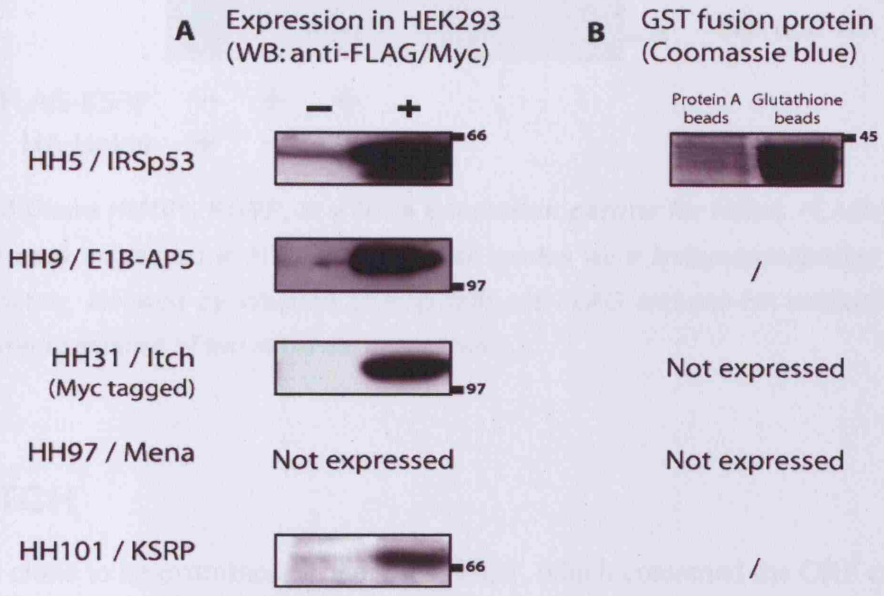

Figure 5.2 Expression of putative Hakai interaction partners. A. *Lysates of HEK293 cells expressing empty vector (left lanes, labelled –) and FLAG-tagged IRSp53, E1B-AP5 or KSRP (right lanes, labelled +) were examined by Western blotting using anti-FLAG antibody. Lysates of HEK293 cells expressing empty vector (–) or Myc-Itch (+) were examined by Western blotting using anti-Myc antibody.* ***B.*** *A GST-fusion of the SH3 domain of IRSp53 was expressed in HEK293 cells, and lysates were pulled down with either protein A beads (left panel) or glutathione beads (right panel). Precipitates were used for SDS-PAGE and the gel stained with Coomassie blue.*

Clones HH70, HH82 and HH99 were marked as lower priority and ultimately were not examined further in this study.

5.2.3 HH101 is a false interaction partner for Hakai

HH101 codes for a K-homology-type splicing regulatory protein (KSRP) (Min et al., 1997). Co-expression of FLAG-KSRP and HA-Hakai in HEK293 cells was followed by precipitation of the lysates with anti-FLAG antibody. Western blotting with anti-FLAG and anti-HA antibodies showed that there was no interaction between these two proteins in HEK293 cells (Figure 5.3). This clone was therefore deemed to be a false interaction partner from the yeast 2-hybrid screen, and was not investigated further.

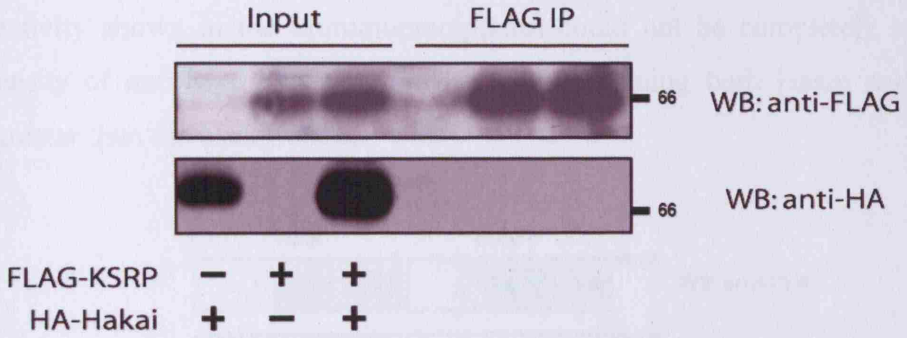

Figure 5.3 Clone HH101, KSRP, is a false interaction partner for Hakai. *FLAG-KSRP and HA-Hakai were expressed in HEK293 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody, followed by Western blotting with anti-FLAG and anti-HA antibodies. These blots are representative of two separate experiments.*

5.3 ITCH

The next clone to be examined further was HH31, which contained the ORF coding for Itch (also known as AIP4), a HECT-type E3 ubiquitin ligase (Courbard et al., 2002). Itch contains an N-terminal protein kinase C-related C2 domain, four WW domains, and a C-terminal HECT (homologous to the E6-associated protein carboxyl terminus) ligase domain (Perry et al., 1998). The HH31 clone contained the sequence for the four WW protein-protein interaction domains. Itch has been shown to interact with a RING-finger E3 ubiquitin ligase, Cbl-c, through its WW domains, and cooperate in the downregulation of the EGF receptor by Cbl-c (Courbard et al., 2002). It was subsequently shown that Itch and another HECT-type E3 ligase, Nedd4, can also

ubiquitinate Cbl RING finger E3 ligases, targeting them for proteasomal degradation (Magnifico et al., 2003). Hakai is structurally homologous to Cbl (Fujita et al., 2002), perhaps indicating the existence of a conserved mechanism of interaction between RING finger ligases and Itch.

5.3.1 Immunoprecipitation between Hakai and Itch

FLAG-Hakai and Myc-Itch were co-expressed in HEK293 cells and lysates were immunoprecipitated with anti-FLAG antibody. Western blotting with anti-FLAG and anti-Myc antibodies confirmed the interaction between Hakai and Itch (Figure 5.4). Despite repeating this experiment under a variety of conditions, including lysing cells in three different buffers (Triton X-100, 0.25% SDS, and RIPA), the background anti-Myc reactivity shown in the immunoprecipitates could not be completely removed. The intensity of anti-Myc reactivity in the lane containing both Hakai and Itch is clearly greater than the background.

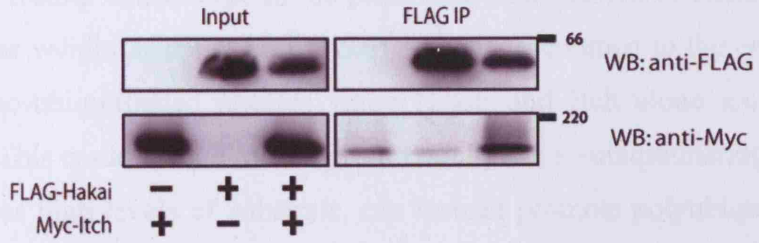

Figure 5.4 Interaction between Hakai and Itch. *FLAG-Hakai and Myc-Itch were expressed in HEK293 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody, followed by Western blotting with anti-FLAG and anti-Myc antibodies. These blots are representative of three separate experiments.*

5.3.2 Itch-dependent modification of Hakai

FLAG-Hakai and Myc-Itch were co-expressed in HEK293 cells and lysates were immunoprecipitated with anti-FLAG antibody. Western blotting of total cell lysates with anti-FLAG antibody shows that in the presence of Itch, Hakai is modified to increase its molecular weight, resulting in an 'upsmear' of staining on the blot (Figure 5.5). This is reminiscent of the pattern of modifications present when a protein is ubiquitinated, which suggests that Itch could be targeting Hakai for ubiquitination.

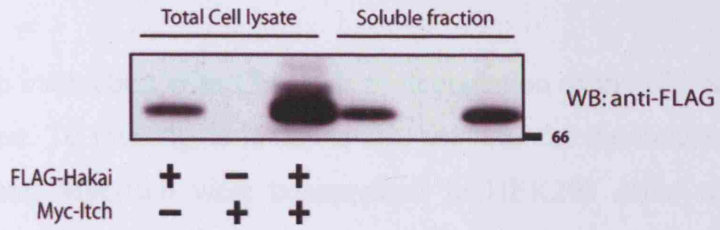

Figure 5.5 Itch dependent modification of Hakai. *FLAG-Hakai and Myc-Itch were expressed in HEK293 cells. Total cell lysates and the soluble fraction that is used for immunoprecipitation were examined by Western blotting using anti-FLAG antibody. This blot is representative of two separate experiments.*

Due to extremely high background, it was not possible to use an anti-ubiquitin antibody to verify the nature of these modifications. An alternative approach was taken, whereby HA-tagged ubiquitin was overexpressed to examine the effect on the Itch-induced modification of Hakai (Figure 5.6). Overexpressing HA-ubiquitin alone did not affect expression of Hakai. However, in the presence of Itch, expression of HA-ubiquitin results in a change in the pattern of modification of Hakai to give more high molecular weight species (Figure 5.6). This is in contrast to the enrichment of a possibly mono-ubiquitinated species when Hakai and Itch alone are co-expressed (Figure 5.6). This could be due to Itch preferentially mono-ubiquitinating Hakai, but in the presence of high levels of substrate, can instead promote polyubiquitination. This result suggests that Itch can ubiquitinate Hakai, and is a further example of a HECT-type E3 ligase targeting a RING-finger type E3 ligase for ubiquitination, in an analogous fashion to the Nedd4/Itch interaction with Cbl (Magnifico et al., 2003).

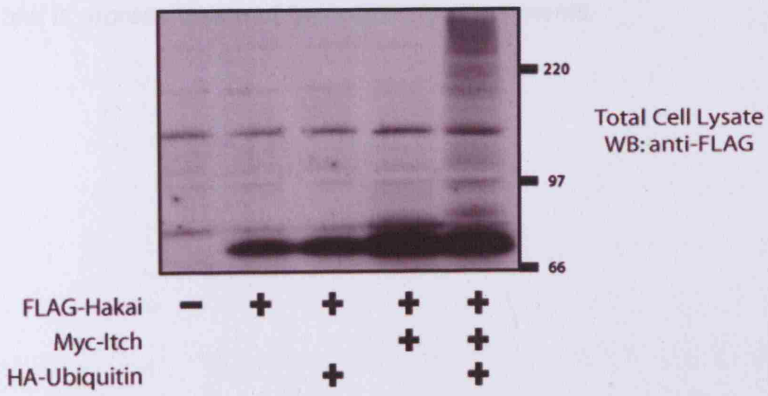

Figure 5.6 Effect of overexpressing Itch and ubiquitin on Hakai. *Total cell lysates of Hek293 cells expressing FLAG-Hakai, Myc-Itch and HA-Ubiquitin were examined by Western blotting using anti-FLAG antibody. This blot is representative of two separate experiments.*

The Nedd4/Itch interaction with Cbl leads to degradation of the Cbl protein following its ubiquitination. To investigate if this is also the case for the Itch/Hakai interaction, FLAG-Hakai and Myc-Itch were coexpressed in HEK293 cells, which were then treated with 20 μM MG132, a proteasome inhibitor, for 6 h prior to lysis. In the presence of a proteasome inhibitor, it was expected that protein degradation would be halted, and an accumulation of the ubiquitinated species would occur. However, levels of Hakai remained constant in MG132-treated cells, compared to untreated cells (Figure 5.7). Thus, it would appear that if Itch is causing the ubiquitination of Hakai, the ubiquitin moiety does not direct Hakai for degradation, but may instead be involved in other signalling pathways.

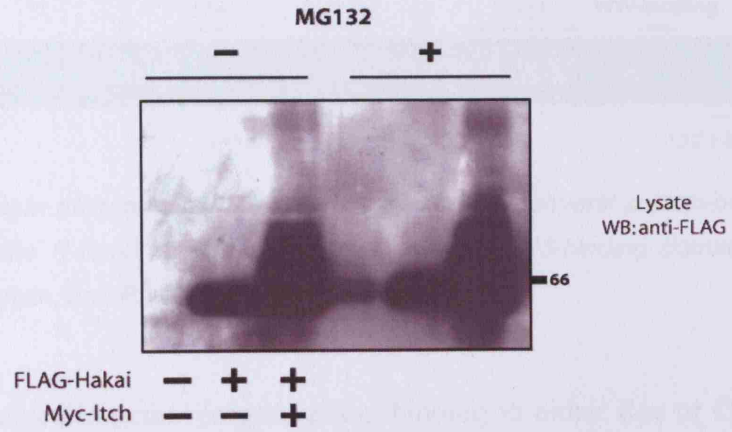

Figure 5.7 Effect of treating cells expressing Hakai and Itch with proteasome inhibitor MG132. *HEK293 cells expressing FLAG-Hakai and Myc-Itch were incubated with 20 μM MG132 for 6 h, followed by lysis and examination by Western blotting using anti-FLAG antibody. This blot is representative of two separate experiments.*

5.4 IRSp53

The third clone to be analysed was HH5, which contained part of the ORF for IRSp53 (Insulin Receptor Substrate p53), including the SH3 domain. IRSp53 (also known as BAP2) was originally identified as an insulin/insulin-like stimulation-dependent tyrosine phosphorylated protein in CHO cells (Yeh et al., 1996). It contains several protein-protein interaction domains, specifically a partial CRIB (Cdc42/Rac interactive binding) domain, SH3-binding domain, SH3 domain, WW-binding domain, and putative PDZ-binding sequence (Figure 5.8; (Okamura-Oho et al., 2001). IRSp53 was deemed to be a likely candidate to bind to the proline-rich C-terminus of Hakai, due to the presence of its SH3 domain.

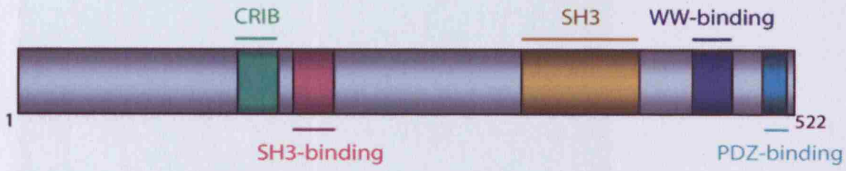

Figure 5.8 Domain structure of IRSp53. *IRSp53 contains several protein-protein interaction domains: from the N-terminus, a partial CRIB domain, SH3-binding domain, SH3 domain, WW-binding domain, and PDZ-binding sequence.*

IRSp53 is involved in actin reorganization, binding to either Rac or Cdc42, which in turn induce the interaction between IRSp53 and WAVE2 or Mena, respectively (Govind et al., 2001; Krugmann et al., 2001; Miki et al., 2000). IRSp53 has been shown to be an important regulator of membrane ruffling (Govind et al., 2001; Krugmann et al., 2001; Miki et al., 2000). Therefore, the interaction between Hakai and IRSp53 could indicate the involvement of Hakai in cell motility. This is particularly interesting for a number of reasons. An involvement in motility would correlate with data that shows an increase in cell scattering of MDCK cells upon the overexpression of Hakai (Fujita et al., 2002). In addition, immunofluorescence data shows that Hakai is found in membrane ruffles of COS-1 cells that have been treated with EGF (Figure 5.9; by kind permission of Y. Fujita), suggesting a role for Hakai in membrane dynamics. Moreover, Hakai may also interact with Mena (clone HH97, Table 5.1), the IRSp53-binding protein, which is located at the tips of filopodia and

promotes actin filament elongation (Cramer, 2002). Unfortunately, the Mena-Hakai interaction could not be verified, thus this line of investigation was not further pursued.

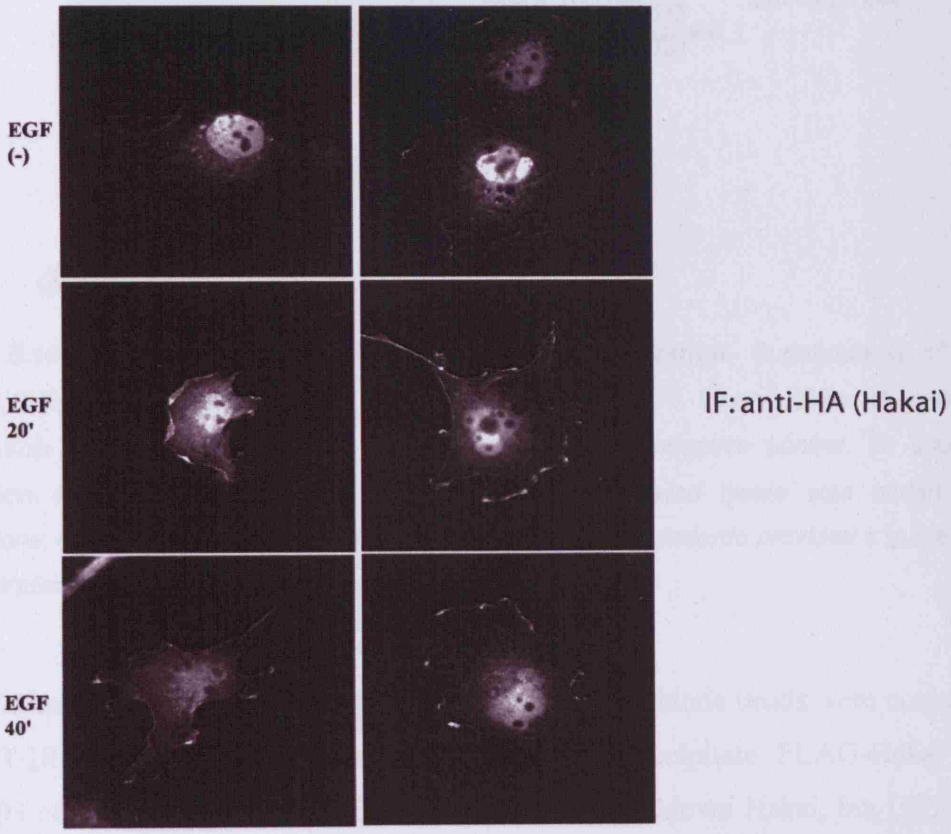

Figure 5.9 Hakai localizes to membrane ruffles in EGF-treated cells. *Immunofluorescent staining of exogenous Hakai. HA-Hakai was transiently transfected in COS-1 cells. Cells were treated with 50 ng/ml EGF for 0, 20 or 40 min prior to fixation. The localisation of Hakai was examined by epifluorescent microscopy using anti-HA antibody.*

5.4.1 IRSp53 SH3 –GST pulldown with Hakai

The fragment of the ORF coding for the SH3 domain of IRSp53 was ligated into pGEX, a vector containing the sequence for Glutathione S-transferase (GST), which allows expression of the GST-fusion protein in *E. coli*. Supernatants of bacteria expressing GST-IRSp53(SH3) were precipitated with glutathione beads and resolved by SDS-PAGE. The gel was coomassie stained and protein levels were estimated by comparison with BSA standards (Figure 5.10). 100 µl of supernatant was estimated to contain approximately 2 µg of protein.

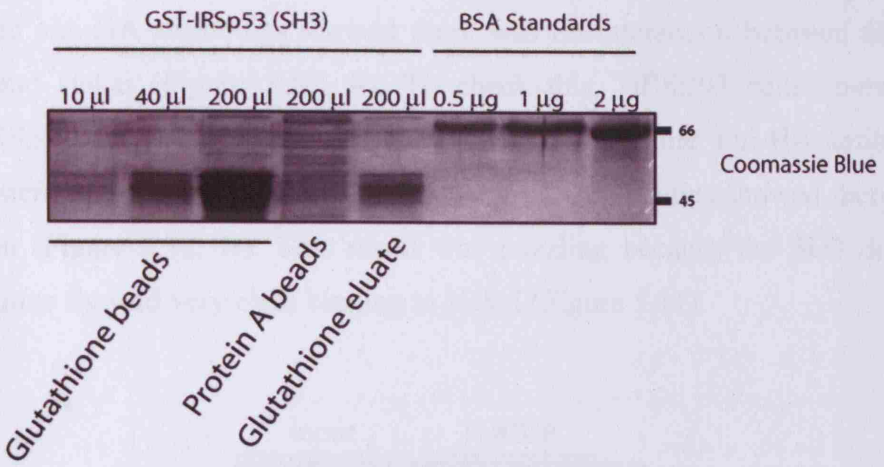

Figure 5.10 Determination of GST-IRSp53(SH3) concentration. *Supernatants of GST-IRSp53(SH3) were precipitated using glutathione beads using 10, 40 and 200 µl of lysate. 200 µl of lysate was precipitated using Protein A beads as a negative control. To show the interaction with the glutathione beads is specific, precipitated lysate was eluted using glutathione. Comparison of the intensity of the bands to BSA standards provided a guide to the concentration of the supernatant.*

HEK293 cells were transfected with FLAG-Hakai. Glutathione beads were conjugated to GST-IRSp53(SH3) or GST, and were used to precipitate FLAG-Hakai from HEK293 cell lysates. GST-IRSp53(SH3) was able to pulldown Hakai, but GST alone was not (Figure 5.11). This suggests that IRSp53 can bind to Hakai through its SH3 domain.

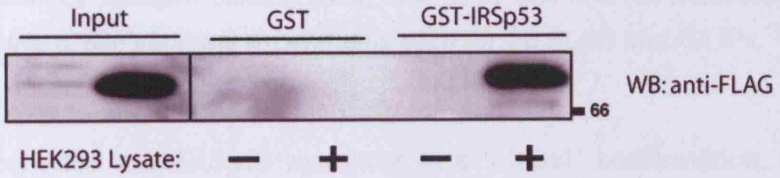

Figure 5.11 Interaction between Hakai and IRSp53(SH3) by a GST pulldown assay. *Lysates of HEK293 cells expressing FLAG-Hakai were pulled down by GST- or GST-IRSp53(SH3)-coated beads and were examined by Western blotting using anti-FLAG antibody. This blot is representative of two separate experiments.*

5.4.2 No interaction of full-length IRSp53 with Hakai

HEK293 cells co-expressing FLAG-IRSp53 and HA-Hakai were immunoprecipitated using anti-FLAG antibody and resolved by SDS-PAGE. Western blotting using anti-

FLAG and anti-HA antibodies showed there was no interaction between full-length IRSp53 and Hakai (Figure 5.12, **A**). To check this, HEK293 cells co-expressing FLAG-IRSp53 and HA-Hakai were immunoprecipitated using anti-HA antibody, but again western blotting using anti-FLAG and anti-HA antibodies showed there was no interaction (Figure 5.12, **B**). This result was puzzling because the SH3 domain of IRSp53 alone showed very clear binding to Hakai (Figure 5.11).

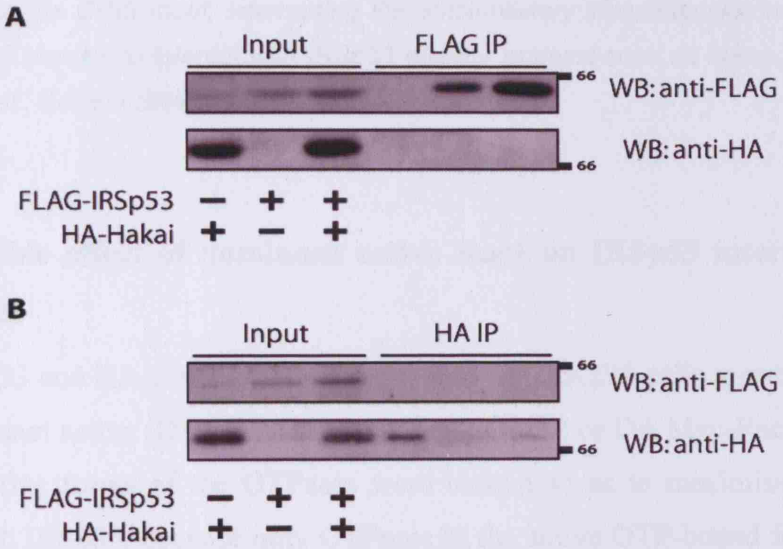

Figure 5.12 No interaction between Hakai and full-length IRSp53. A. *Lysates of HEK293 cells expressing FLAG-IRSp53 and HA-Hakai were immunoprecipitated with anti-FLAG antibody, followed by Western blotting using anti-FLAG and anti-HA antibodies.* ***B.*** *Lysates of HEK293 cells expressing FLAG-IRSp53 and HA-Hakai were immunoprecipitated with anti-HA antibody, followed by Western blotting using anti-FLAG and anti-HA antibodies. These blots are representative of two separate experiments each for the FLAG and HA IPs.*

It has been reported that IRSp53 can exist in a 'closed' confirmation, with its SH3 domain unavailable for protein-protein interactions due to an auto-inhibitory interaction of the CRIB domain with the N-terminus (Figure 5.13; (Krugmann et al., 2001). Binding of the CRIB domain of IRSp53 to GTP-loaded Cdc42 can 'open' the structure allowing an interaction between IRSp53 and Mena (Krugmann et al., 2001). The results in Figures 5.11 and 5.12 suggest there may be a similar mechanism for the interaction between IRSp53 and Hakai. Therefore, co-expression of small GTPases with Hakai and IRSp53 was carried out to investigate the possibility that an interaction between Hakai and IRSp53 may occur under these conditions.

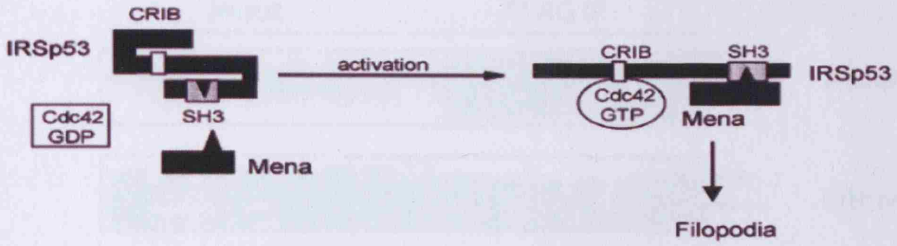

Figure 5.13 A model of the regulation of IRSp53. *In its resting state, IRSp53 is in a 'closed' conformation, masking the SH3 domain. In response to a stimulatory signal, GTP-bound Cdc42 binds to the CRIB motif, interrupting the autoinhibitory intramolecular interaction. This allows the SH3 domain to interact with IRSp53 effector proteins such as Mena. Diagram from Krugmann et al., Current Biology, 2001.*

5.4.3 Possible effect of dominant active Rac1 on IRSp53 interaction with Hakai

FLAG-IRSp53 and HA-Hakai were co-expressed in HEK293 cells together with Myc tagged dominant active (DA) Cdc42 (Myc-Cdc42 Q61L) or DA Myc-Rac1 G12V. The dominant active forms of the GTPases were chosen so as to maximise the binding potential with IRSp53, because only GTPases in the active GTP-bound form can bind to the CRIB domain of target proteins (Burbelo et al., 1995). Lysates were precipitated using anti-FLAG antibody, and Western blotted with anti-FLAG, anti-HA and anti-Myc antibodies. The co-expression of DA Cdc42 Q61L had no effect (Figure 5.14). An interaction between IRSp53 and Hakai was observed in the presence of DA Rac1 G12V (Figure 5.14), but this data was not reproducibly observed. An alternative approach would be to co-transfect wildtype Rac1 or Cdc42 with IRSp53 and Hakai, as it may be that the cycling between GDP and GTP bound states of the GTPase is required for the interaction to occur.

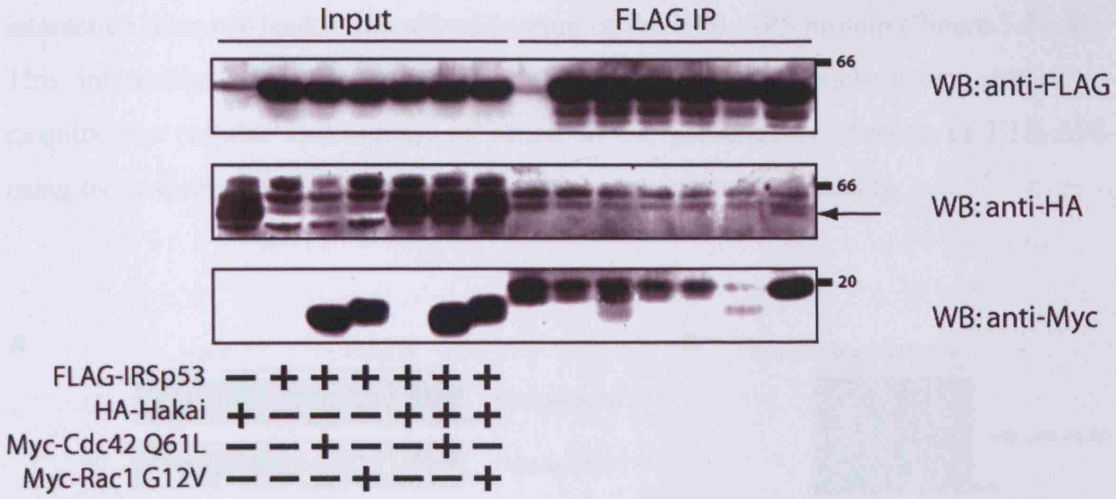

Figure 5.14 Possible interaction between Hakai and IRSp53 in the presence of dominant active Rac1. *Lysates of HEK293 cells expressing FLAG-IRSp53, HA-Hakai, constitutively active Myc-Cdc42 Q61L or Myc-Rac1 G12V were precipitated using anti-FLAG antibody and examined by Western blotting using anti-FLAG, anti-HA and anti-Myc antibodies. Arrow indicates band in precipitate that corresponds to molecular weight of HA-Hakai.*

5.5 E1B-AP5

E1B-AP5 (Adenovirus early region 1B-associated protein 5), clone HH9 from the yeast 2-hybrid screen (Table 5.1), is a member of the heterogenous nuclear ribonucleoprotein (hnRNP) family (Gabler et al., 1998). It is involved in RNA transport, and has also recently been shown to interact with p53, inhibiting the transcriptional activity of p53 in UV radiation experiments (Barral et al., 2005). The suggestion of an association between Hakai and a nuclear protein is interesting because nuclear accumulation of Hakai has been observed in a number of cell lines (A. Figueroa & Y. Fujita, unpublished data). This could point to a nuclear role for Hakai.

5.5.1 E1B-AP5 immunoprecipitation with Hakai

FLAG-E1B-AP5 and HA-Hakai were co-expressed in HEK293 cells, and lysates were precipitated using anti-FLAG antibody. Western blotting with anti-FLAG and anti-HA antibodies confirmed the interaction between Hakai and E1B-AP5 (Figure 5.15, **A**). Examining the anti-FLAG Western blot of the total cell lysate did not show any potential modification of E1B-AP5 in the presence of Hakai, suggesting that this

interaction does not lead to the ubiquitination of the E1B-AP5 protein (Figure 5.15, **B**). This interaction could be examined further by immunohistochemistry studies to examine the cellular localisation of Hakai in the presence or absence of E1B-AP5 using overexpression of E1B-AP5 or siRNA knockdown, respectively.

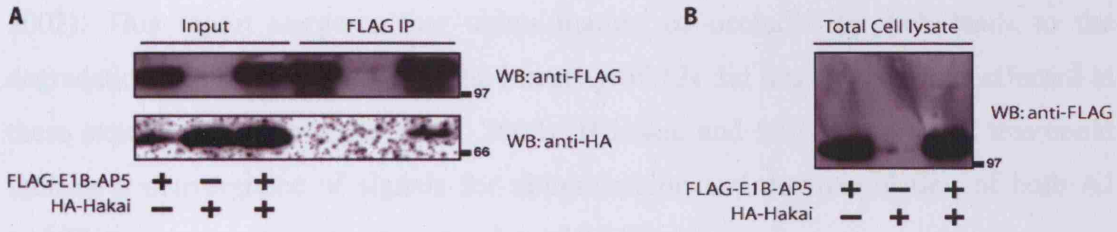

Figure 5.15 Interaction between Hakai and E1B-AP5. A. *Lysates of HEK293 cells expressing FLAG-E1B-AP5 and HA-Hakai were immunoprecipitated using anti-FLAG antibody, followed by Western blotting using anti-FLAG and anti-HA antibodies.* ***B.*** *Total cell lysate examined by Western blotting using anti-FLAG antibody.*

5.6 DISCUSSION

The results in this chapter indicate that Hakai could have several more interaction partners in addition to E-cadherin. A number of interesting lines of investigation have been opened up by the possibility that Hakai could be involved in further levels of regulation by ubiquitination with Itch; actin remodelling events with IRSp53 and Mena; and nuclear processes with E1B-AP5. Some of the implications of these preliminary results are discussed below.

5.6.1 Hakai and the ubiquitin system

Hakai is an E3 ubiquitin ligase that is structurally related to c-Cbl. So far, Hakai has only been shown to target the E-cadherin complex for ubiquitination and internalisation (Fujita et al., 2002). The suggestion here that Hakai can interact with the HECT-type E3 ubiquitin ligase Itch, which is also known to bind to c-Cbl (Magnifico et al., 2003), indicates that Hakai could be involved in additional levels of regulation of E3 ligase activity and receptor tyrosine kinase signalling.

Itch has been shown to have many other interaction partners in the cell, including Notch, JunB, and Human enhancer of filamentation 1 (HEF1) (Feng et al., 2004; Gao et al., 2004; Qiu et al., 2000). This opens up avenues of investigation into the possible contribution Hakai could be having by acting in concert (or antagonistically) with Itch. A particularly interesting candidate to investigate is occludin, a tight junction (TJ) specific protein, which was shown to be a functional target of Itch (Traweger et al., 2002). This report suggests that ubiquitination of occludin by Itch leads to the degradation of occludin, although the integrity of TJs did not appear to be affected in these experiments (Traweger et al., 2002). If Hakai and Itch act together, this could indicate a convergence of signals for ubiquitination and downregulation of both AJ and TJ components by the same complex of E3 ligases.

5.6.2 Hakai and actin remodelling

An intriguing possibility has been raised by the observation that Hakai can bind both IRSp53 and Mena, proteins that form a complex upon Cdc42 activation, and which are involved in cytoskeletal dynamics. This suggests that ubiquitination of the IRSp53-Mena complex could be occurring, which may point to an additional level of complexity in the regulation of actin remodelling. The interplay between cell-cell adhesion and cell motility could also be affected by this dual specificity of Hakai for E-cadherin and IRSp53/Mena.

Cdc42 can bind to IRSp53 causing the localisation of IRSp53 to filamentous actin (Govind et al., 2001). This leads to reorganisation of the F-actin cytoskeleton, with loss of stress fibres and formation of filopodia (Govind et al., 2001). Further, Krugmann and colleagues have reported that Cdc42 binding to IRSp53 allows the interaction between IRSp53 and Mena, and that it is this complex which induces the formation of filopodia downstream of Cdc42 activity (Krugmann et al., 2001). Subsequently, work from the Small lab indicates that IRSp53 can also be found co-localized with WAVE2 at the tips of protruding lamellipodia as well as filopodia (Nakagawa et al., 2003). This interaction with WAVE2 is downstream of Rac binding to the IRSp53 CRIB domain. Whether IRSp53 could tie WAVE2 and Mena into a complex is the subject of speculation (Nakagawa et al., 2003). Data presented in this thesis gives an indication that this complex could be further regulated by Hakai.

Indeed, immunofluorescence data of overexpressed Hakai in COS-1 cells treated with EGF shows that Hakai can be found in membrane ruffles (Figure 5. 8) (Y. Fujita, unpublished data), further supporting this hypothesis.

Comparing Hakai to the structurally related protein c-Cbl, it seems likely that, in addition to its enzymatic activity as an E3 ligase, Hakai could also be functioning as an adapter protein linking proteins in cytoskeletal dynamics. c-Cbl has been shown to behave as an adapter, binding to a number of proteins (reviewed in (Swaminathan and Tsygankov, 2006), including those involved in the regulation of Rho-family GTPases and the cytoskeleton: Vav, a Rho guanine nucleotide exchange factor (GEF), binds directly to tyrosine 700 of c-Cbl (Marengere et al., 1997; Miura-Shimura et al., 2003), while another RhoGEF, DOCK180, can bind to Crk, also a c-Cbl-binding protein (Chiang et al., 2001; Hasegawa et al., 1996). In addition, recruitment of PI3K to c-Cbl can lead to activation of Rac in transformed fibroblasts (Teckchandani et al., 2005). Interestingly, as well as promoting the formation of complexes to regulate the actin cytoskeleton, c-Cbl can also negatively regulate Vav through ubiquitination and subsequent degradation of ubiquitinated Vav (Miura-Shimura et al., 2003). It is tempting to speculate that Hakai could also be acting in a similar way as a 'double-edged sword' to play an adaptor role on the one hand, facilitating the formation of an IRSp53-Mena complex at membrane protrusions, and then ubiquitinating components of the complex to target the signalling platform for disassembly/degradation. Temporal separation of these two processes would make E3 ligases excellent scaffolds for responses to cell activation followed by speedy dampening of the signal.

5.6.3 Nuclear functions for Hakai

Hakai has been observed in the nucleus in a number of cell lines (A. Figueroa & Y. Fujita, unpublished data). The existence of nuclear targets is therefore likely. The suggestion here that Hakai can interact with an RNA-binding protein, E1B-AP5, points to a possible role in transcriptional or translational regulation of gene expression. This is well known to be the case for other E3 ligases, for example Mdm2, which binds to the transactivation domain of p53, preventing transcriptional activation, and also promoting the nuclear export of p53 (Boyd et al., 2000; Geyer et al., 2000; Wu et al., 1993). The recent report from Barral and colleagues that describes an interaction

between E1B-AP5 and p53 (Barral et al., 2005), raises an interesting possibility that Hakai could perhaps form part of this complex and impact on transcriptional events downstream of p53 activation. Reporter assays using a luciferase construct containing p53 DNA-binding motifs, expressed in the presence of p53, E1B-AP5 or Hakai, or both E1B-AP5 and Hakai together, would address this proposal. In other work from our laboratory, Hakai has been shown to bind to PSF (polypyrimidine tract-binding protein-associated splicing factor) in HEK293 cells and this interaction leads to an increase in the mRNA-binding capability of PSF (A. Figueroa, unpublished data), suggesting that Hakai can indeed functionally interact with nuclear proteins.

5.6.4 Regulation of Hakai activity

The mechanisms that regulate Hakai binding to its targets and promoting ubiquitination have not been investigated in this study, but are important to consider. From the initial report on Hakai function in E-cadherin internalisation, it is clear that availability of substrate (in this case, tyrosine phosphorylated E-cadherin) is one factor which limits Hakai function (Fujita et al., 2002). There is also some evidence reported in this study to suggest that ubiquitination by Itch can occur, and further work to clarify whether Hakai function is perturbed by this modification will provide insight into the regulation of Hakai. Ubiquitin ligases are also known to be regulated by factors such as compartmentalisation, post-translational modifications, and interactions with other effectors (Dikic et al., 2003). For example, phosphorylation is a very important mechanism to regulate Cbl activity (Dikic et al., 2003). For Cbl to interact with SH2 domain-containing proteins, it must be tyrosine phosphorylated (Dikic et al., 2003); tyrosine phosphatases such as SHP-1 and SHP-2 are implicated in the regulation of Cbl signalling in phagocytosis and in chemotaxis, respectively (Chernock et al., 2001; Kant et al., 2002). In contrast, serine phosphorylation of Cbl is a negative regulatory mechanism (Liu et al., 1999). In addition, Cbl is targeted by a protein called Spry2, which binds to the RING finger domain, thus preventing ubiquitination of the EGF receptor and potentiating EGFR-mediated MAP kinase activation (Egan et al., 2002; Wong et al., 2002; Wong et al., 2001). Whether such mechanisms are in place for regulation of Hakai function should be addressed.

CHAPTER 6

FINAL DISCUSSION

6.1 COORDINATION OF SIGNALLING FROM THE ADHERENS JUNCTION

Work presented in this thesis suggests that p120 has a role to play in neuronal differentiation during development, through its interaction with the transcription factor Glis2 (Figure 6.1). Sequestration by E-cadherin at the AJ prevents p120 from interacting with Glis2. This is also the case for the p120/Kaiso interaction (Kelly et al., 2004; Roczniak-Ferguson and Reynolds, 2003). These data suggest that upon the disruption of AJs in response to a particular trigger, p120 becomes available to take part in other signalling pathways. How p120 affects the transcriptional regulation activity of Glis2 is not clear. A model is put forward in Figure 6.2 to suggest how p120 affects the promoter regulation of Glis2 according to the results of the chick neural tube experiments described in Chapter 4.

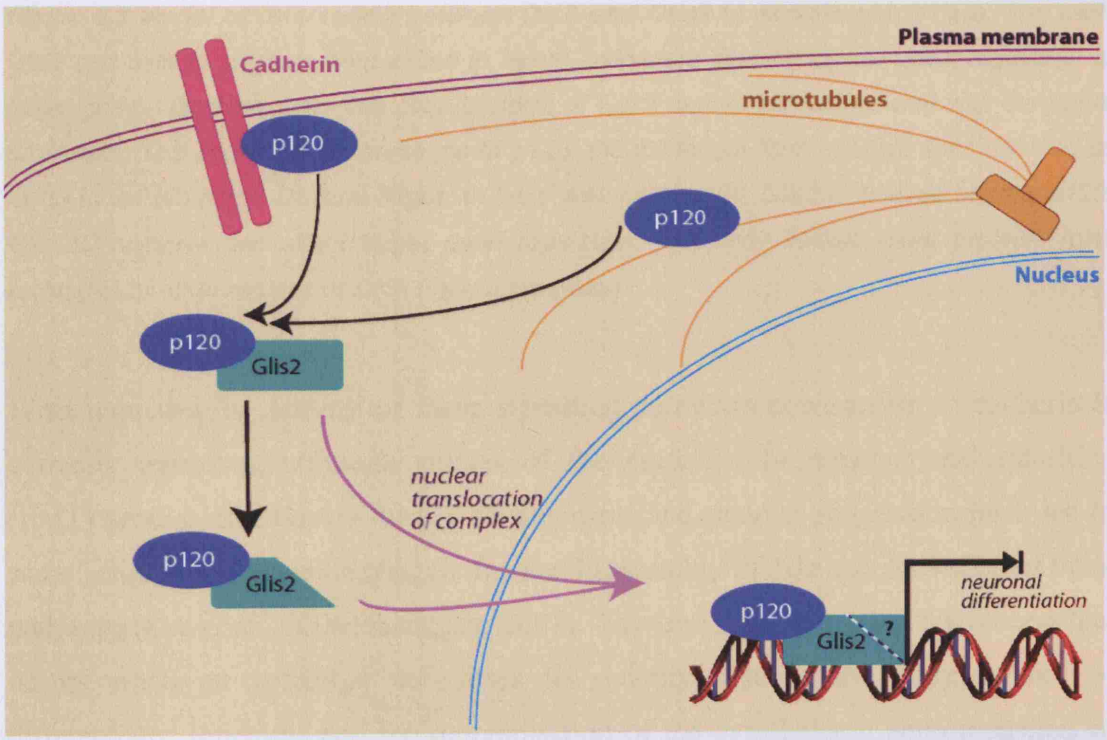

Figure 6.1 p120 interaction with Glis2. *p120 binds to Glis2 and causes its cleavage. p120 sequestration by E-cadherin or microtubules inhibits the p120-induced cleavage of Glis2. In the presence of Glis2 and Src, p120 is translocated to the nucleus. Glis2 and Glis2 ΔC can bind to DNA. Glis2 can affect neuronal differentiation, as can Glis2 ΔC in the presence of p120. The targets of Glis2 (and Glis2 ΔC with p120) are currently unknown.*

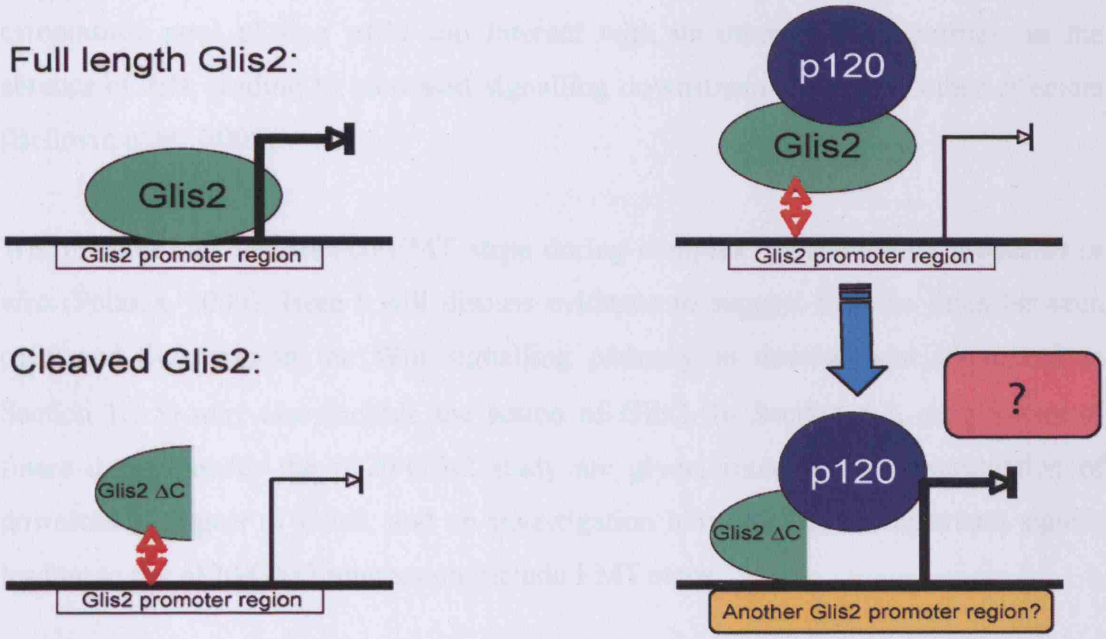

Figure 6.2 Model of interaction between DNA and Glis2 to explain chick data. Top Left: *Glis2 can bind to cognate sequences in target promoters causing up- or down-regulation of target genes.* ***Bottom Left:*** *The cleaved form of Glis2 cannot interact so well with the target promoters.* ***Top Right:*** *In the presence of p120, the full-length form of Glis2 does not bind so avidly to the promoter.* ***Bottom Right:*** *In the presence of p120, Glis2 is cleaved and the p120-Glis ΔC complex can affect target gene regulation. This may involve other proteins (pink rectangle) or other regions of DNA (yellow promoter).*

What regulates the activity of these signalling pathways downstream of cadherin is currently unknown, although studies of the epithelial to mesenchymal transition (EMT) process that occurs during development and tumour progression have led to some insights into possible triggers for the disassembly of AJs and activation of other pathways (Lee et al., 2006; Savagner, 2001). The term EMT describes the process that occurs when an epithelial cell loses its polarity, due to the disappearance of differentiated junctions and the reorganisation of the cytoskeleton and organelles, in response to an appropriate signal (Thiery, 2003). These cells are highly motile, and processes such as neural crest cell migration and gastrulation rely on this conversion (Thiery, 2003). In cancer, highly invasive carcinoma cells show a loss of epithelial characteristics and a consequent increase in cell motility (Lee et al., 2006; Savagner,

2001), thus demonstrating that aberrant signalling pathways inside the cell can cause EMT without an extracellular trigger. A defining characteristic of EMT is the loss of E-cadherin (Thiery, 2003), and so it is possible to speculate that the resultant cytoplasmic pool of free p120 can interact with its other binding partners in the absence of AJs, leading to increased signalling downstream of p120's other effectors (Bellovin et al., 2005).

Wnt proteins can also induce EMT steps during complex differentiation processes *in vivo* (Polakis, 2000). Here I will discuss evidence to suggest that the links between p120 and β-catenin in the Wnt signalling pathway in development (described in Section 1.3.5) may also include the action of Glis2. In Section 6.3, suggestions of future directions for the p120/Glis2 study are given, including the examination of downstream targets of Glis2, and an investigation into whether the upstream signals leading to the p120/Glis2 interaction include EMT steps.

6.2 CONVERGENCE OF SIGNALLING FROM CADHERIN ON THE WNT PATHWAY

Developmental processes that require Wnt signalling such as segmentation, gastrulation and central nervous system patterning all require EMT processes. During these processes epithelial cell subpopulations actively downregulate cell-cell adhesion and move into new microenvironments where they differentiate into distinct cell types (Polakis, 2000; Savagner, 2001). As described in Section 1.2.2, β-catenin has a well characterized role in transducing signals from Wnt into the nucleus. β-catenin binding to TCF/LEF derepresses transcription of Wnt target genes (Hlsken and Behrens, 2000). In the absence of β-catenin, TCFs repress transcription by interacting with the co-repressors Groucho and CtBP (Cavallo et al., 1998; Roose et al., 1998; Valenta et al., 2003). Recent evidence implicates p120 and Kaiso in further modulation of Wnt signalling to the *Siamois* promoter (Park et al., 2005). These findings raise the interesting possibility that these two related catenins function in parallel to mediate cadherin-associated regulation of gene expression.

Interestingly, a recent study from the Jetten lab shows an interaction between Glis2 and CtBP1 (Kim et al., 2005), one of the co-repressors that interacts with TCF in the absence of Wnt signals (Valenta et al., 2003). In this study, it appears that Glis2 is part of a co-repressor complex, involving the additional recruitment of histone deacetylases (Kim et al., 2005). In the light of the Kaiso-*Siamois* data, it is possible that this points to further interaction between p120 and the β-catenin/Wnt signalling pathway. Identification of p120/Glis2 gene targets in the future will address this hypothesis.

How p120 may act downstream of Wnt receptor activation at the cell surface is currently under investigation. In contrast to β-catenin, p120 is stable in the cytosol, and is not targeted for degradation by the proteasome. Its signalling activity must be controlled at another level – for example, regulated nuclear translocation, and availability of binding to Kaiso or Glis2. Whether Glis2 and Kaiso compete for binding of p120 will be interesting to investigate. The contribution of Src phosphorylation of p120 will also be very interesting to investigate, as kinases are key regulators of Wnt signalling, for example GSK3β phosphorylation of β-catenin and the inhibition of GSK3β by phosphorylated Dvl (Cadigan and Liu, 2006).

Furthermore, whether cadherins can influence p120/Kaiso or p120/Glis2 regulation of target genes is important to investigate. In the case of β-catenin, it is known that in certain circumstances cadherin binding can inhibit its TCF-dependent nuclear signalling function (Gottardi et al., 2001). Conversely, presence of Wnt signals can lead to a conformational change in β-catenin that favours TCF rather than cadherin binding (Gottardi and Gumbiner, 2004). There may also be similar mechanisms of regulation of p120/transcription factor signalling, as it has been reported that overexpression of cadherin can recruit cytoplasmic p120 to the plasma membrane, and also, changes in expression of different p120 isoforms in tumour cells can be brought about by changes in cadherin expression (Reynolds and Roczniak-Ferguson, 2004). Whether p120 Arm domains are shielded by intra-molecular interactions precluding binding of specific partners has yet to be investigated. In addition, post-translational modification is known to have a role in β-catenin choices between cadherin or TCF association. Phosphorylation of Tyrosine 142 of β-catenin favours binding of the nuclear protein BCL9-2 and precludes the interaction with α-catenin, helping to

modulate the switch between the adhesive and transcriptional function of β-catenin (Brembeck et al., 2004). Data presented in this thesis showing that Src stimulates p120-induced Glis2 cleavage is highly suggestive of a role for phosphorylation in directing nuclear signalling outcomes downstream of p120.

A recent paper from the McCrea group sheds some light on how the p120/Kaiso pathway is linked to Wnt signalling pathways in *Xenopus*, by the discovery that p120 binds to Frodo, a functional regulator of Dishevelled (Park et al., 2006). This interaction enhances the p120-mediated relief of Kaiso repression of *xWnt11* (Park et al., 2006). Further examination of these pathways in mammals will be interesting to compare, given the lack of obvious defects in Kaiso null mice (Prokhortchouk et al., 2006). In addition, the fact that p120 affects Rho GTPases signalling (Section 1.3.3, Introduction) could indicate a possible involvement in non-canonical Wnt signalling (non-canonical Wnt signals are transduced through Rho family GTPases rather than via β-catenin; Cadigan and Liu, 2006) and this has not yet been addressed.

Ultimately, the developmental or physiological significance of coordinate regulation of Wnt target genes by β-catenin and p120 needs to be elucidated. The suggestion that cadherins can modulate transcription of Wnt target genes through two effectors points to the possibility that signalling downstream of cadherin could fine tune transcriptional outcomes in a different fashion to the classical canonical β-catenin-directed signalling.

6.3 FUTURE DIRECTIONS

6.3.1 Identifying Glis2 target genes

A pressing question is whether p120 can modulate Glis2-mediated regulation of specific target genes. However, little is known about the Glis2 DNA binding sites and/or target genes. One approach would be to look at the GLI response element (GLI RE), which Glis2 has been shown to bind to *in vitro* (Lamar et al., 2001). Initial reports from our collaborators indicate that Glis2 affects transcription from the GLI RE in a luciferase reporter assay but the effect is very minor and the presence of p120 does not affect the response (F. Ulloa, unpublished results). Electrophoretic mobility shift assays (EMSA) might shed more light on this question. Indeed, since Glis2 did

not appear to significantly affect GLI signalling in the chick neural tube (Section 4.4), it seems likely that Glis2 binds other regions of DNA, and so chromatin IPs (ChIP) using a 'shotgun' library of short DNA sequences would identify further Glis2 targets. Whether Glis2 ΔC binds to the same targets, or exhibits different affinities to full-length Glis2 is also of great interest.

Despite the data presented in this thesis indicating that p120 does not affect the ability of Glis2 to bind to DNA *in vitro* (Section 4.3.1), it is still possible that p120 could modulate the transcriptional activity of Glis2 through some other mechanism, perhaps by obscuring the binding of a co-repressor or shielding a phosphorylation site. Investigating whether Glis2 is phosphorylated, either by Src itself, or another kinase, will help to address this question. The construction of pseudo-phosphorylated or non-phosphorylatable mutants of Glis2 could then be used to further examine the mechanism of p120 binding, cleavage and Glis2 DNA-binding properties.

6.3.2 Identifying the physiological stimulus for the p120/Glis2 interaction

The trigger for the p120/Glis2 interaction is as yet unidentified. Data presented here indicates that p120 must be free in the cytosol to interact with Glis2. Hence, any stimulus that promotes the accumulation of p120 in the cytosol could be implicated. Furthermore, Src activity increases the interaction between p120 and Glis2 and potentiates the p120-induced cleavage of Glis2. During morphogenesis and tumour progression, EMT signals lead to the partial dissociation of AJs, and Src family kinases (SFKs) are implicated in this process (D'Souza-Schorey, 2005; Frame, 2002; Lee et al., 2006).

In a recent study, a colon carcinoma cell line, LIM1863, was used to examine the expression and function of p120 during EMT (Bellovin et al., 2005). The LIM1863 line undergoes EMT upon co-stimulation with transforming growth factor (TGF)-β1 and tumour necrosis factor (TNF)-α (Bates and Mercurio, 2003). Bellovin and colleagues show that p120 expression persists after E-cadherin downregulation after the onset of the EMT, and its localisation shifts from cell-cell junctions to the cytoplasm (Bellovin et al., 2005). These data suggest that p120 could be able to interact with Glis2 under these conditions.

Elevated expression and/or activation of RTKs or of non-receptor tyrosine kinases is often linked to acquisition of an EMT and the resultant migratory and invasive cancer cell phenotype (Savagner, 2001). SFKs co-localize with E-cadherin at sites of cell-cell contact in non-migrating epithelial cells (Frame, 2002). As described in Section 1.2.1, the role of Src in targeting p120 has not yet been well delineated, but it seems likely that activating Src-mediated tyrosine phosphorylation of p120 leads to disassembly of AJs (Frame, 2002). One piece of corroborative evidence for this is found in KM12C colon carcinoma cells where expression of constitutively active Src induces an obvious EMT. This EMT is characterized by enhanced assembly of dynamic integrin-mediated focal-adhesion-like structures, induction of vimentin expression and concomitant deregulation of cell-cell adhesions and endocytosis of E-cadherin (Avizienyte et al., 2004; Avizienyte et al., 2002). Thus it is possible to speculate that induction of EMT that leads to activation of Src at cell-cell contacts can concomitantly cause the dissociation of p120 from the cadherin JMD and enhance the interaction between p120 and Glis2. Mimicking the induction of EMT in a cell line such as the LIM1863 or KM12C colon carcinoma cell lines mentioned above, in the presence or absence of Src inhibitors and testing for the p120 interaction with Glis2 by immunoprecipitation, would test this theory.

A weakness of the data presented in this thesis is the reliance on overexpression systems in cell lines that are of limited physiological relevance. Repetition of the experiments characterising the p120-induced cleavage of Glis2 using endogenous proteins in epithelial cell systems would benefit the work by providing more information about the dual roles of p120 at AJs and in complex with Glis2. Demonstration of the interaction of p120 with Glis2 in primary cells would provide confirmation of the relevance of this interaction, and provide further cause to investigate signalling targets of this complex.

With regard to the recent evidence conflating p120 and β-catenin signalling pathways downstream of Wnt activation (Park et al., 2006; Park et al., 2005), it will be extremely interesting to investigate whether the p120/Glis2 interaction has an impact on Glis2 binding to CtBP1, and what the downstream transcriptional outcomes may be, if identification of Glis2 targets uncovers Wnt-responsive genes. CtBP1 represses

transcription from the Wnt target gene Axin2/Conductin (Valenta et al., 2003), and so this would be a good starting point with which to investigate the effect of p120/Glis2 on transcriptional regulation.

In conclusion, the potential for further roles of p120 in the nucleus emphasizes the importance of cadherin-based signals in development and cancer. Given the evidence that upon downregulation of cadherin, free cytoplasmic/nuclear p120 can increase the invasive potential of tumours (van Hengel and van Roy, 2006), further delineation of nuclear targets of p120 remains an important goal.

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